DEVELOPMENT OF GENE THERAPY FOR THE TREATMENT OF ADENOSINE DEAMINASE DEFICIENCY

BY

EMMA KATIA MADELEINE BJÖRKEGREN

Molecular Immunology Unit
Institute of Child Health
University College London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Adenosine deaminase (ADA) severe combined immunodeficiency (SCID) is a life-threatening condition resulting from lack of the ADA enzyme. Consequences include immunodeficiency and non-immunological symptoms such as neurological abnormalities. Bone marrow transplantation (BMT) from a haploidentical donor usually results in complete restoration of immune function. However, the majority of patients do not have a matched donor and are therefore treated with enzyme replacement therapy (PEG-ADA). This treatment is not always fully effective, it is expensive and needs to be administered throughout life. Gene therapy is an alternative treatment, and previous trials for ADA deficiency have shown that it can significantly improve immunological function.

Immune recovery was assessed in three ADA-SCID patients treated with PEG-ADA by analysis of lymphocyte counts and emergence of naïve T cells. One patient was not responding well to PEG-ADA and was enrolled in a Phase I clinical gene therapy trial. A gammaretroviral vector encoding ADA was constructed and tested extensively on cell lines and patient cells and a $CD34^+$ cell transduction protocol was optimised. The gene therapy procedure was based on previous successful trials, and involved withdrawal of PEG-ADA prior to treatment to provide selective growth advantage for transduced cells, and mild conditioning to encourage engraftment. Assessments of immune function were then performed in a similar manner to patients treated with PEG-ADA.

Recent evidence from studies of ADA deficiency indicates that it is a multi-organ disease. However, gene therapy using $CD34^+$ cells may only correct the immunodeficiency without ameliorating non-immunological symptoms. Hence, studies were performed to develop systemic gene therapy for ADA-SCID, involving the use of $CD34^+$ cells and mesenchymal stem cells (MSCs). MSCs were isolated from bone marrow, and their multipotential nature was assessed prior to and following gene transfer using a cloned ADA lentiviral vector. Transduced MSCs maintained their ability to undergo differentiation and transgene expression was not affected by this.

These clinical and preclinical *in vitro* studies demonstrate that gene therapy holds therapeutic potential for treatment of ADA-SCID.

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ABBREVIATIONS

AAV	adeno associated virus
ADA	adenosine deaminase
ADP	adenosine diphosphate
ALC	absolute lymphocyte count
BD	Becton Dickinson
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
CB	cord blood
CBU-E	colony burst unit-erythroid
CD	cluster of differentiation
cDNA	complimentary DNA
CDP	cytosine diphosphate
CDR	complimentary determining region
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming unit
CFU-G	colony forming unit-granulocyte
CFU-GM	colony forming unit-granulocyte macrophage
CFU-M	colony forming unit-macrophage
cPPT	central polypurine tract
CY	phycoerythrin-cyanine 5
dAdo	deoxy adenosine
dATP	deoxy adenosine triphosphate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynuclotide triphosphate
DTT	dithio-DL-threitol
EBV	Epstein Barr virus
ECAAC	European collections of cell cultures
ECL	enhanced chemiluminescence
ECM	extracellular matrix

EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anaemia virus
FACS	fluorescence activated cell sorting
FC	flow cytometry
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GALV	gibbon ape leukaemia virus
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GMP	good manufacturing practise
GTAC	Gene Therapy Advisory Committee
GVHD	graft versus host disease
hFlt3	human *fms*-like tyrosine kinase 3
hIL3	human interleukin 3
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
HSC	haematopoietic stem cell
hSCF	human stem cell factor
HSV	herpes simplex virus
hTPO	human thrombopoietin
IHC	immunohistochemistry
IL	interleukin
IP	immunophenotyping
IVIG	intravenous immunoglobulin
Kb	kilobases
LASN	LTR-ADA-SV40-NeoR
LB	Lennox-L broth
LCL	lymphoblastoid cell line
LDL	low density lipoprotein
LNGFR	low affinity nerve growth factor receptor

LTBMC	long term bone marrow culture
LTR	long terminal repeat
MAPC	multipotent adult progenitor cell
MESV	murine embryonic stem cell virus
MFI	mean fluorescence intensity
MNC	mononuclear cells
MOI	multiplicity of infection
MSC	mesenchymal stem cell
MUD	matched unrelated donor
NeuN	neuron specific nuclear protein
NIH	National Institute of Health
NK	natural killer cell
NSE	neuron-specific enolase
PBMC	peripheral blood mononuclear cell
pBS	plasmid bluescript
PBS	phosphate buffered saline
PCMV	PCC4 cell passaged murine sarcoma virus
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PEI	polyethyleneimine
Pen-Strep	penicillin-streptomycin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
PMSF	phenyl methyl sulfonyl fluoride
PNP	purine nucleoside phosphorylase
qPCR	quantitative PCR
RAG	recombinase activating gene
RBC	red blood cell
RCL	replication competent lentivirus
RCR	replication competent retrovirus
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	reverse transcription

SA-PE	streptavidin-phycoerythrin
SCC	single cell clone
SCID	severe combined immunodeficiency
SFFV	spleen focus forming virus
SIN	self-inactivating
TBA	tetrabutylammonium hydrogen sulphate acetate
TCA	trichloroacetic acid
TGFβ3	transforming growth factor beta 3
TREC	T cell receptor gene rearrangement excision circle
UCB	umbilical cord blood
UDP	uracil diphosphate
VCAM	vascular cell adhesion molecule-1
VSV-G	vesicular stomatitis virus glycoprotein
v/v	volume per volume
WB	whole blood
WPRE	woodchuck post-transcriptional regulatory element
w/v	weight per volume

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INTRODUCTION

1.1 ADENOSINE DEAMINASE DEFICIENCY

1.1.1 Severe Combined Immunodeficiency

Severe Combined Immunodeficiency (SCID) is a rare heterogeneous disorder that results in profound defects of both cellular and humoral immunity. Almost 100 different primary immunodeficiencies have been documented (Primary immunodeficiency diseases, Report of an IUIS Scientific Committee, 1999), of which SCID is the most severe. Symptoms include failure to thrive, lymphopenia, fungal, viral and bacterial infections, and is lethal at an early age unless treated. Several genetic defects leading to the clinical and immunological phenotype of SCID have been identified, the most common being the X-linked disorder, accounting for 40-50% of all SCID cases (Noguchi *et al.*, 1993). Adenosine deaminase (ADA) deficiency is an autosomal recessive disease, which accounts for approximately 20% of all cases of SCID, based on studies reviewed by Hirschhorn (1990a & b), although recent European data suggests that the incidence may be 11% of the SCID cases (Figure 1.1). ADA deficiency was first identified in the early 1970s (Giblett *et al.*, 1972), and is caused by a mutation in the gene for the ADA metabolic enzyme. It is a rare disease, affecting only between 1 in a million to 1 in 100,000 children. The child often presents in the first few months of life with failure to thrive, and suffers from a variety of infections. Other less common causes of SCID include deficiencies in the enzyme purine nucleoside phosphorylase (PNP), involved in the same metabolic pathway as ADA, and deficiencies in the recombination activating genes (RAG) 1 and 2 important in the generation of antigen specific B and T cell receptors.

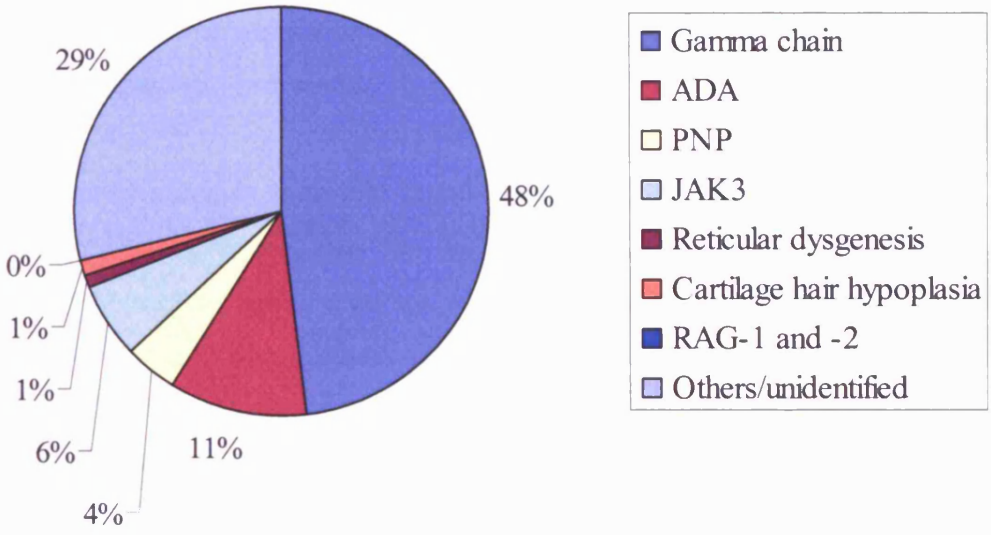


Figure 1.1. Prevalence of different forms of SCID.

1.1.2 Clinical Aspects of ADA Deficient SCID

The first and most severe clinical manifestation of ADA deficiency is immunodeficiency, which leaves the patient susceptible to infections. ADA-SCID patients have an absence of B, T and NK cells (Buckley *et al.*, 1997), resulting in a complete absence in cellular and humoral immunity. ADA deficient patients often present with persistent diarrhoea, failure to thrive and candidiasis (Morgan *et al.*, 1987) as well as other opportunistic infections (viral, fungal, bacterial and protozoan). Patients with ADA deficiency are among the SCID patients which are most seriously ill, and often present in the first few months of life. The average age of presentation has been calculated to be 4.4 months (Arredondo-Vega *et al.*, 1998), which includes the average delay in diagnosis of about 2 months after initial infective problems. This delay in diagnosis is often contributed to by the presence of some immunoglobulins at birth due to the maternal IgGs. However, 1-2 months after the birth, hypogammaglobulinaemia becomes apparent.

Lymphopenia and elevation of toxic substrates such as dATP (discussed below) have been found to be present pre-natally (Linch *et al.*,1984). Moreover, ADA deficient patients do not have isoagglutinins and the immunoglobulins, especially IgA and IgM, are low or absent. As a result, the antibody responses to T-dependent antigens are severely impaired.

ADA deficiency not only results in immunodeficiency, but has been found to be a multi-organ disease, which may be as a result of ADA being a ubiquitous housekeeping enzyme (reviewed by Hirschhorn *et al.*, 1999). The systemic abnormalities include autoimmunity, skeletal abnormalities (Cederbaum *et al.*, 1976), cupping and flaring of costochondral junctions, renal mesangial sclerosis (Ratech *et al.*, 1985) and hepatic abnormalities (Bollinger *et al.*, 1996). Autopsies of early onset disease patients have revealed abnormalities in the spleen, lymph nodes, gut and thymus, (reviewed by Hirschhorn *et al.*, 1979d; Borzy *et al.*, 1979; Ratech *et al.*, 1985, 1989). Similarly to other cases of SCID, thymic biopsies and autopsies have revealed absent or small dysplastic organs, and no or low numbers of lymphocytes are found in thymus, spleen or lymph nodes (Ratech *et al.*, 1989). Similar non-immunological symptoms are also found in ADA deficient mice including pulmonary and intestinal defects as well as hepatocyte degeneration (Migchielsen *et al.*, 1995 & 1996; Wakamiya *et al.*, 1995; Blackburn *et al.*, 1998 and 2000a & b; Aldrich *et al.*, 2003) (discussed in more detail in section 1.1.6). However, due to often overwhelming infections in ADA deficient patients, it is difficult to determine whether these non-immunological abnormalities are primarily due to ADA deficiency. With improved survival as a result of bone marrow transplantations (BMT) and enzyme replacement therapy, it is likely that more symptoms will be discovered that were not previously apparent in the young children.

ADA patients have also been found to have significant neurological abnormalities including spasticity, head lag, movement disorders and sensori-neuronal deafness (Hirschhorn *et al.*, 1980; Tanaka *et al.*, 1996; Albuquerque & Gaspar, 2004). A neuropsychological study also observed behavioural difficulties including hyperactivity, attention deficit and aggressive tendencies in transplant patients (Rogers *et al.*, 2001). It has been found that high levels of ADA are expressed in the brain (Hirschhorn *et al.*, 1978), and that there is a significant correlation between

dATP levels at diagnosis and total IQ (Rogers *et al.*, 2001). The neurological abnormalities may also be due to increased interactions between adenosine and adenosine A_1 receptors in nervous tissue, as there was found to be an improvement in neurological abnormalities concomitant with the lowering of toxic levels of metabolites as a result of therapeutic measures (Hirschhorn *et al.*, 1980).

Disease Type	**Phenotype**
Neonatal/Infantile Onset	85-90%. Indistinguishable from other forms of SCID, except bony abnormalities in some patients.
Delayed/Late Onset	Diagnosis after 2nd year of life. Antibodies present, weaken later. May have recurrent bacterial sinopulmonary infections, lymphopenia & autoimmunity.
Adult Onset	Very rare. Late onset symptoms and persistent viral warts, lymphopenia.
Partial ADA Deficiency	Often lack erythrocyte ADA – retain normal immune function.

Table 1.1. Clinical phenotypes in ADA deficient SCID. A wide range of phenotypes have been observed, from early onset to adult onset.

The genotype in ADA deficiency results in a spectrum of clinical phenotypes, where the age at onset of disease differs (Table 1.1). As some of this variation occurred within families, it is thought that environmental differences such as early infections or early treatments may also influence the severity of the disease. The most common phenotype of ADA deficiency is the early onset "classical" SCID. These patients present with failure to thrive, lymphopenia, and absence of cellular and humoral immune system as a result of the lack of B, T, and NK cells. Unless treated, this form of ADA deficiency is fatal at an early age, usually due to overwhelming infections. Some of the early onset ADA deficient patients, unlike other SCID patients, also display non-immunological symptoms such as skeletal abnormalities of the costochondral junction (Wolfson & Cross, 1975; Cederbaum *et al.*, 1976). Approximately 10-15% of ADA deficient patients have a delayed onset disease, diagnosed beyond the first year of life, and show an initially milder disease

progression (Hirschhorn, 1979a, b & c; Cohen *et al.*, 1979; Geffner *et al.*, 1986; Levy *et al.*, 1988; Santisteban *et al.*, 1993; Umetsu *et al.*, 1994). These patients produce some immunoglobulins, however these are essentially non-specific antibodies (reviewed by Hirschhorn, 1999). In some of these patients serious infections start at 2 to 3 years of age, but in others sinus and upper respiratory infections, including bacterial pneumonia, are observed in slightly younger patients.

Late onset ADA deficiency is diagnosed from around 3 to 15 years of age or even later in life (Geffner *et al.*, 1986; Morgan *et al.*, 1987; Levy *et al.*, 1988; Shovlin *et al.*, 1993; Ozsahin et al., 1997). These patients have numerous clinical manifestations, including lymphopenia (in particular T cell lymphopenia) and recurrent sinopulmonary bacterial infections, usually pneumonia caused by *Streptococcus pneumoniae*. They may lack IgG2 but have hyper-IgE and may have eosinophilia and autoimmunity. This group of patients may be under-diagnosed, but would show a gradual immunologic and clinical deterioration over time.

A very rare form of adult onset disease was reported in two siblings by Shovlin *et al.* in 1993. These two patients started to display a variety of symptoms in late adolescence, including persistent viral warts and immune-mediated thrombocytopenic purpura as well as all the symptoms displayed in the late onset disease.

Partial ADA deficiency has been identified as a result of a screening programme, and has been found to be due to the lack of ADA in erythrocytes. The majority of these individuals have a normal immune function as they display 5-80% of the normal ADA activity in non-erythroid cells (Cohen *et al.*, 1978; reviewed by Hirschhorn, 1979b; Perignon *et al.*, 1980; Borkowsky *et al.*, 1980; Schmalstieg *et al.*, 1983; Daddona *et al.*, 1983; Hirschhorn & Ellenbogen, 1986; Hart *et al.*, 1986; reviewed by Hirschhorn, 1990a & b). The lack of ADA in only erythrocytes is thought to be due to an unstable enzyme in cells which cannot synthesise new protein. Partial ADA deficiency has also been diagnosed as a result of somatic mosaicism of the mutations in the ADA gene (Hirschhorn *et al.*, 1994 & 1996; Arredondo-Vega *et al.*, 2002). In one of these cases described by Hirschhorn *et al.*, it resulted from a very rare spontaneous reversion mutation to normal. The patient described by Arredondo-Vega

et al., showed a second mutation which resulted in the normalisation of the splicing pattern.

Overproduction of ADA in erythrocytes has been identified in some patients with an inherited form of haemolytic anaemia (Valentine *et al.*, 1977; Miwa *et al.*, 1978; Perignon *et al.*, 1982). Their ADA levels in lymphocytes were found to be normal, hence their immune system was functional. The anaemia was thought to be caused by low levels of erythroid ATP, due to increased adenosine monophosphate metabolism and decreased adenine nucleotide synthesis from adenosine (Perignon *et al.*, 1982). Although the molecular basis for this condition is not known, it is thought that the enhanced ADA expression seen in red blood cells (RBCs) is due to a mutation in an ADA gene regulatory element.

1.1.3 Molecular Pathology of ADA Deficiency

ADA (EC3.5.4.4) is a metabolic enzyme of the purine salvage pathway, catalysing the irreversible, hydrolytic deamination of adenosine and 2'-deoxyadenosine (dAdo) to inosine and 2'-deoxyinosine respectively as depicted in Figure 1.2. It is a ubiquitous enzyme expressed at varying levels in different tissues, and is particularly abundant in tissues where there is a high turnover of cells and where a large amount of DNA needs to be salvaged. Although ADA is a general housekeeping enzyme, the main effect of its absence is on the immune system, which may be due to high levels of ADA being expressed in the thymus and in other lymphoid tissues, signifying its importance in these cell types. It is, however, believed that due to the ubiquitous nature of this enzyme, a lack of ADA also leads to non-immunological symptoms not usually seen in other SCID patients, such as hepatic (Bollinger *et al.*, 1996) and neurological abnormalities (Hirschhorn *et al.*, 1980; Rogers *et al.*, 2001).

Deficiencies in metabolic enzymes usually lead to disease either as a result of a toxic build-up of enzyme substrates, or as a result of the lack of enzyme products. In the case of ADA deficiency, it is the former which appears to be true because there are alternative pathways which can compensate for the lack of ADA products (reviewed by Hirschhorn, 1999). It has been observed that the extent of the metabolic

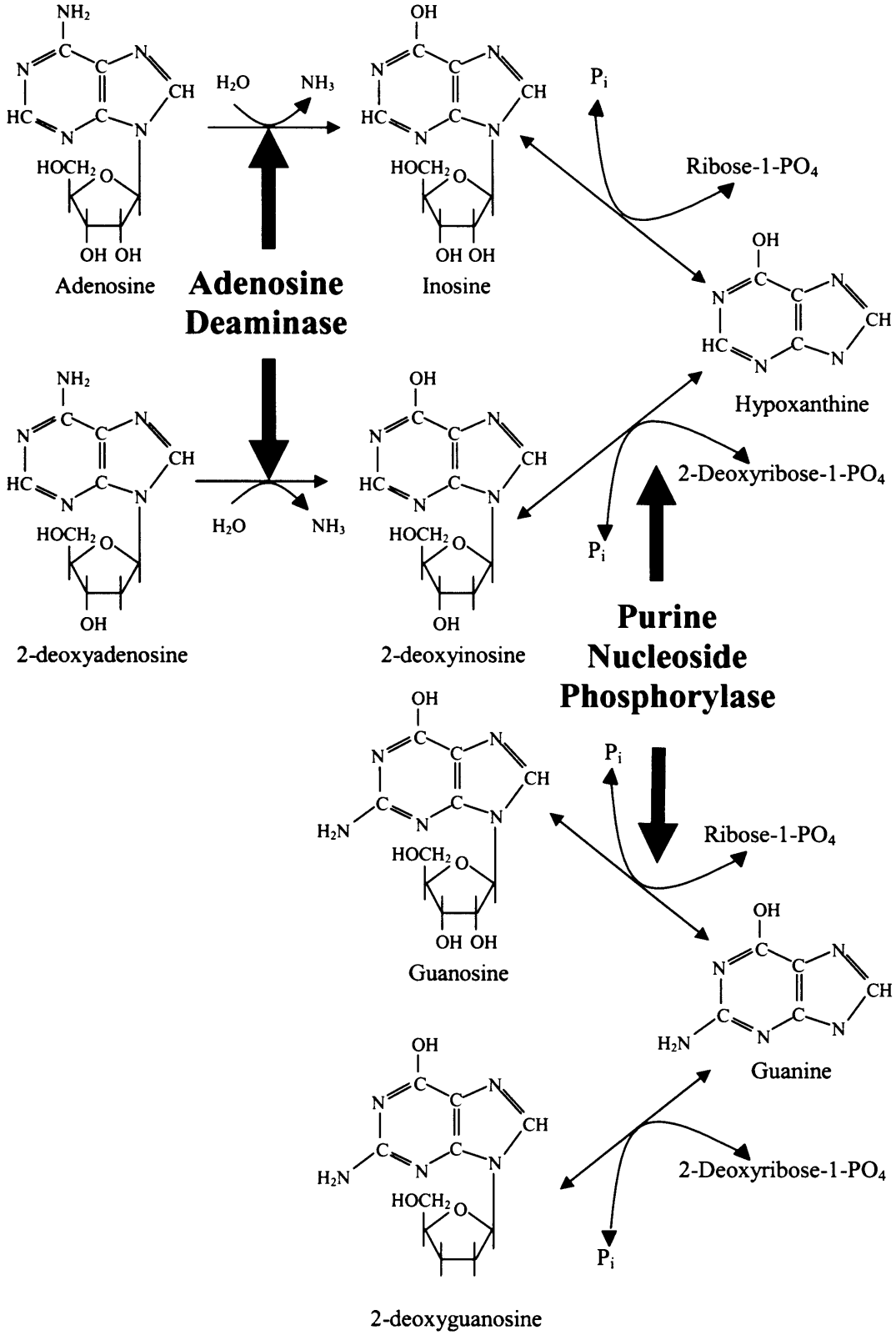


Figure 1.2. Reactions catalysed by ADA and purine nucleoside phosphorylase. ADA converts adenosine and 2-deoxyadenosine to inosine and deoxyinosine respectively in a deamination reaction. These products are then further converted to hypoxanthine by the enzyme purine nucleoside phosphorylase as part of the purine salvage pathway. (Adapted from Hershfield and Mitchell, 2001).

abnormalities is significantly related to the severity of the disease (Morgan *et al.*, 1987). ADA deficiency results in elevated toxic levels of the ADA substrates adenosine and deoxyadenosine (dAdo) in the plasma and elevated dAdo levels in the urine. Levels of dATP are also increased as this is produced from the phosphorylation of the increased levels of d-adenosine by the enzyme d-cytidine kinase or adenosine kinase in a usually minor pathway (Figure 1.3). Increased dATP is trapped in cells and is no longer in equilibrium with the extracellular fluid. It is this accumulation of dATP which appears to be the main cause of cellular toxicity, based on *in vitro* experiments. dATP inhibits the enzyme ribonucleotide reductase which is important in DNA replication. Ribonucleotide reductase catalyses the reduction of ADP, GDP, CDP and UDP to form dNTPs, in a rigorously controlled reaction, which maintains the dNTP pools at levels sufficient for only a few minutes of DNA replication. Hence, the inactivation of this enzyme by dATP leads to the inhibition of DNA replication, and consequently cellular toxicity. This effect is mainly apparent in cells of high turnover, such as thymocytes and lymphocytes, which may explain the selective toxicity to these cells in ADA deficient patients (Henderson *et al.*, 1980; Ullman *et al.*, 1980; Waddell & Ullman, 1983; Kubota *et al.*, 1984; Albert *et al.*, 1984; Mann & Fox, 1986). Moreover, dATP tends to accumulate in erythrocytes and lymphocytes as a result of a higher rate of phosphorylation of dAdenosine compared with the rate of dephosphorylation and the subsequent trapping of dATP. A further way in which dATP may cause toxicity is by activating the caspase cascade in thymocytes and peripheral blood cells, which results in apoptosis in both dividing and non-dividing cells and could therefore explain the lack of thymocytes in ADA deficiency (Liu *et al.*, 1996; Li *et al.*, 1997). The substrate dAdenosine is derived mainly from the breakdown of DNA, hence it is expected to be highly expressed in areas of cell death such as the thymus where the T cells undergo apoptosis as a part of the selection procedure. On the basis of *in vitro* experiments, it has been proposed that high levels of dAdo result in DNA strand breaks (Brox *et al.*, 1984). Elevated levels of dAdo, as well as adenosine, can also result in the inactivation of S-adenosyl homocysteine hydrolase (SAHH), with less than 5% normal activity usually being observed (Hershfield *et al.*, 1979a & b; Hirschhorn *et al.*, 1981; Hershfield *et al.*, 1985; Hershfield *et al.*, 1987; Santisteban *et al.*, 1993; Hershfield *et al.*, 1993). This leads to an increased level of adenosyl homocystein which inhibits methylation dependent reactions such as gene expression, RNA splicing and protein synthesis

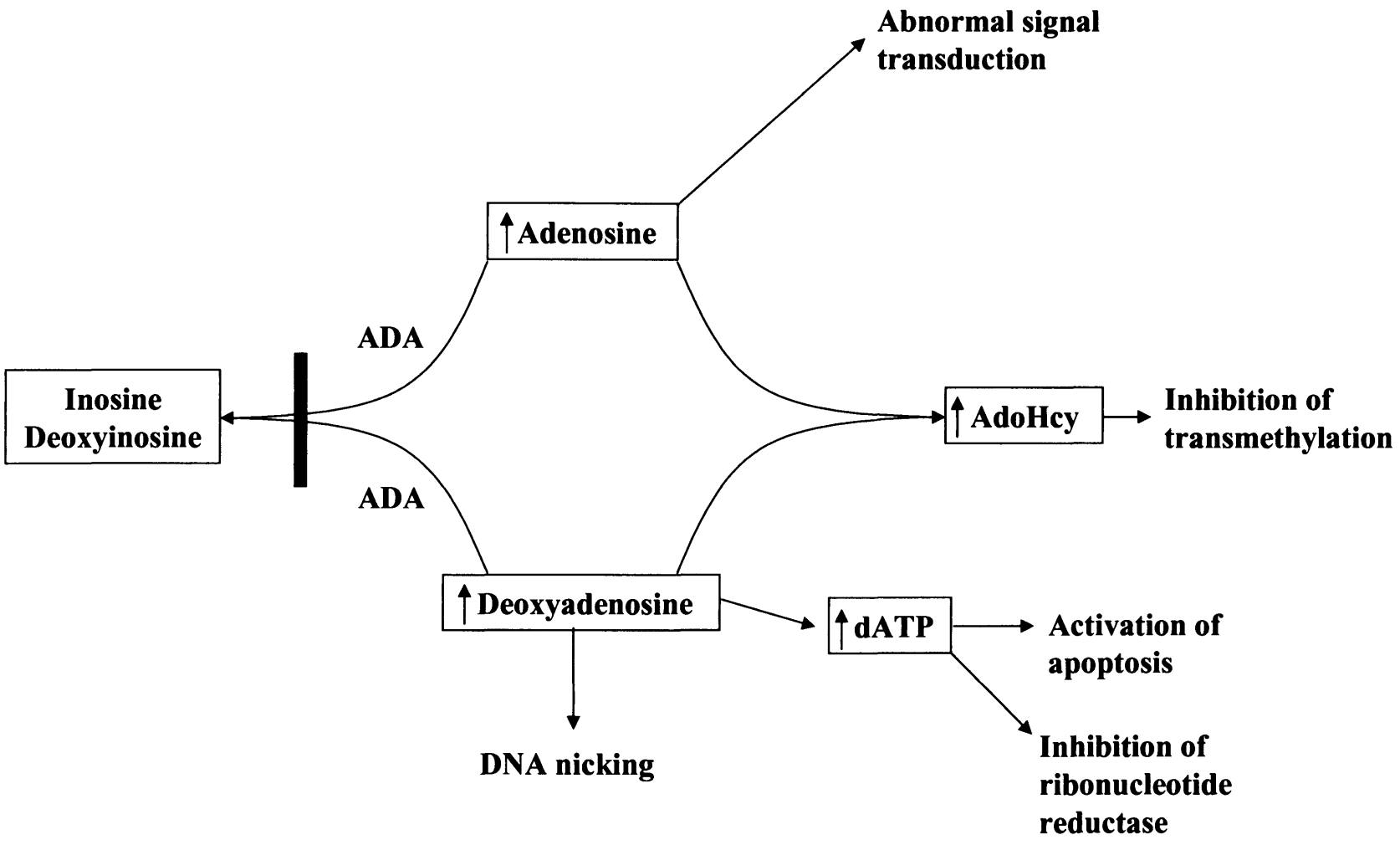


Figure 1.3. Toxicity of ADA substrates. It is thought that the absence of ADA leads to cellular toxicity due to the build up of toxic levels of ADA substrates as shown in this schematic diagram. AdoHcy, adenosyl homocystein. (Adapted from Hershfield and Mitchell, 2001).

(Kredich & Martin, 1977; Abeles *et al.*, 1982). However, dAdo is thought to be less of a cause of cell toxicity than dATP, because elevated SAH has not been demonstrated *in vivo* in patients, and inhibition of SAHH is still observed in bone marrow transplanted patients who have normal immune functions.

Adenosine is a component of RNA and the energy storing compound ATP. It is, like dAdo, derived from the breakdown of ATP in dying cells. Adenosine is thought to exhibit its main effects through the G-protein associated adenosine A1 and A2 receptors leading to abnormal signal transduction, although these effects have not been shown to be clinically significant (Kizaki *et al.*, 1988; Kizaki *et al.*, 1990; Apasov *et al.*, 1997). Adenosine may be the ADA substrate with the least effect on cell toxicity.

It is therefore apparent that the accumulation of the ADA substrates leads to cell toxicity, and the concentrations of these metabolites correlate with the severity of the disease. However, the reasons behind the selective toxicity to lymphocytes have not yet been fully elucidated. It has been proposed that the destruction of mainly the immune system can be attributed to the fact that ADA activity levels vary over 100-fold in the different tissues of the body. Because the immune cells display high levels of ADA activity, it appears logical that this is the system which is mainly affected. However, this theory fails to explain why other tissues that show high ADA activity levels are not affected, such as the gastrointestinal system. Hence, it is clear that more research needs to be performed to determine why ADA deficiency mainly affects the immune system.

1.1.4 ADA – Gene, Protein and Enzyme Activity

The ADA gene is located on the long arm of chromosome 20 (20q12-q13.1) (Tischfield *et al.*, 1974; Mohandas *et al.*, 1980; Mohandas *et al.*, 1984; Jhanwar *et al.*, 1989). The human ADA gene has been cloned (Valerio *et al.*, 1983; Valerio *et al.*, 1985; Wiginton *et al.*, 1983) and sequenced (Wiginton *et al.*, 1986). The total length of the gene is 32,040 base pairs, made up of 12 exons and 11 introns (Figure 1.4). The cDNA is approximately 1.5kb long, containing a coding region of 1089 nucleotides.

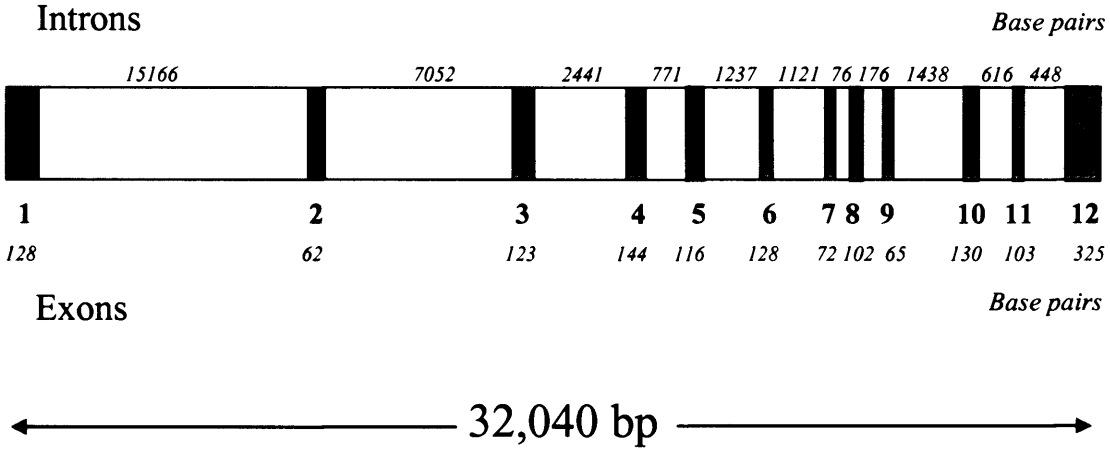


Figure 1.4. Schematic diagram of the human ADA gene (not to scale). The human gene for ADA is made up of 32,040 nucleotides. It is composed of 12 exons which give rise to a 1.5kb cDNA, containing a 1089bp open reading frame. (Adapted from Hershfield and Mitchell, 2001).

The 40 kDa human protein is a monomer consisting of 363 amino acids (Daddona & Kelley, 1977; Schrader *et al.*, 1976; Schrader & Stacy, 1977; Wiginton *et al.*, 1981). The three-dimensional structure has been solved for the murine ADA (which is 83% identical to the human protein) by X-ray crystallography, and shows a parallel α/β barrel structure with a central zinc atom in the active site (Wilson *et al.*, 1991; Sharff *et al.*, 1992).

Adenosine deaminase is found mainly in the cytoplasm of the cells, however a small proportion can also be found in some cell types in a complex with two molecules of CD26 (Nishihara *et al.*, 1973; Van der Weyden & Kelley, 1976; Daddona & Kelley, 1978; Schrader *et al.*, 1979; Daddona & Kelley, 1980). It is thought that the ADA expressed on the cell surface of cells may regulate local concentrations of adenosine which affect the stimulatory and inhibitory adenosine receptors.

The ADA protein is found in all tissues, although its levels of expression vary greatly over a 10^3-10^4 fold range (Brady & O'Donnovan, 1965; Van der Weyden & Kelley, 1976). In addition, ADA is also developmentally regulated, where in humans a decrease in ADA expression is seen from immature cortical thymocytes to medullary thymocytes and mature T-lymphocytes, as well as in the maturation of B cells

(Chechik *et al.*, 1981; Chechik *et al.*, 1984). ADA activity is thought to be important in all cell types, and the highest activity levels are detected in the thymus and in other lymphoid tissues, particularly immature T cells, in the brain and in the gastrointestinal tract. The lowest ADA activity levels are found in erythrocytes (Edwards *et al.*, 1971; Van der Weyden & Kelley, 1976; Adams & Harkness, 1976; Carson *et al.*, 1977; Hirschhorn *et al.*, 1978; Witte *et al.*, 1991). Enzyme activity in many ADA-SCID patients is effectively undetectable, and if any activity is observed, this is usually due to the minor activity of a non-relevant isozyme that exhibits ADA activity and is not affected in ADA deficiency. Later onset patients have generally retained 2-5% of the enzyme activity (Shovlin *et al.*, 1993). In most ADA deficient patients analysed it has been determined that the mutation in the ADA enzyme has resulted in an unstable protein, hence explaining the complete lack of ADA activity. Approximately half of the mutations identified in ADA deficient patients do not express enzyme activity. Of the remainder, one group of mutations result in 0.5-2% normal activity, a second group results in 3-6% and a third group results in ADA activity of 10-80% of normal. The reasons for these ranges in ADA activity may be due to the instability of the mutant protein, disruption of the central zinc-interacting amino acids or changes of amino acids in the active site (reviewed by Hirschhorn, 1993).

There appears to be a good correlation between levels of ADA activity and severity of disease. A lower enzyme activity is usually associated with a more severe disease progress. As carriers of the mutated ADA gene appear to have normal immune function, the amount of ADA activity levels needed to maintain a functional immune system are thought to be quite low, possibly as low as 5% of normal (Hirschhorn, 1999). Some children who present with late onset ADA-SCID have been found to have trace amounts of ADA activity, in the region of 1-2% of normal activity levels.

1.1.5 Mutational Analysis of ADA

More than 70 different mutations have been identified by Hirschhorn (Hirschhorn *et al.*, 1992) and many others (reviewed by Hershfield and Mitchell, 2001) that result in ADA deficiency. A large proportion of these are point mutations (single base pair changes), although there are also some deletions (ranging from single nucleotide to

large deletions), insertions (insertion of one or more nucleotides), splice mutations (often single nucleotide changes which result in abnormal splicing), as well as a few nonsense mutations (point mutations which introduce a pre-mature stop codon and therefore shorten the protein). From the mutations analysed in ADA deficiency, it appears that a small number of mutations may be responsible for the disease in several patients. This could be due to one of two reasons, either due to founder effect or due to mutational hot spots especially at CpG dinucleotides. Several missense mutations (point mutations resulting in an amino acid change) at mutational hot spots have been reported in ADA-SCID individuals, which would lead to either unstable or inactive protein. The gene is also rich in Alu sequences within the introns, which have been shown to be involved in causing deletions in the ADA gene.

Genotype/phenotype correlations have been studied to a large extent, however it has been made difficult mainly by the fact that ADA deficiency is such a rare disease and that most patients are compound heterozygotes, carrying two different mutations. These studies are also complicated because environment and linked or unlinked modifying genes can affect the genotype/phenotype relationship. One difficulty was how to define the clinical phenotype. This could be done by the age of onset or the extent of immunological abnormalities; however the cellular immune dysfunctions can be greatly modified by the time and nature of infectious agents the patient encounters. Phenotype could therefore also be defined by the amount of residual ADA activity, by the extent of accumulation of ADA substrates or by the response to different medications. Despite these difficulties, some correlations can still be defined. A patient homozygous for the Arg261Gly mutation and a patient with a mutation in the downstream codon were both found to have very severe phenotypes. These codons were discovered to be in the region of the substrate binding site which has been found to be conserved through evolution. It is therefore thought that these two mutations are candidates for markers of severe disease (Hirschhorn *et al.*, 1993). Other mutations have been found to confer a milder disease progress. Some patients who responded better than others to a crude form of enzyme replacement therapy, partial exchange transfusions, were found to carry certain mutations. Ala329Val is one mutation which may be associated with a milder phenotype and has been found to code for 0.5-2% of normal ADA activity. Similarly, Arg211His was found in two patients to encode for some ADA activity, and two mutations at codon 101 were also

identified in two other patients (Hirschhorn *et al.*, 1993). One of the mutations at codon 101 is thought to code for some ADA activity in T cells, hence it is likely to be a less severe mutation. Certain other mutations (e.g. Ala215Thr, Arg156His) were also found in several ADA deficient patients with a delayed onset but not in patients with early onset disease (Hirschhorn *et al.*, 1990c; Santisteban *et al.*, 1993; Ozsahin *et al.*, 1997). One of the delayed onset patients was found to have a mutation that allowed for the expression of 1% of normal ADA levels (Hirschhorn *et al.*, 1993). Analysis by Hershfield in 2003, revealed that 90% of the patients with an early onset disease had alleles expressing less than 0.05% of normal ADA activity. He also showed that only one of the early onset disease patients had one allele which resulted in slightly higher ADA activity. Moreover, the vast majority of the alleles known to confer 0.1-11% of normal ADA activity, were associated with delayed, adult onset or partial phenotypes. Hence it is clear that despite the difficulties in discerning genotype/phenotype relationships, some mutations have been identified which result in a more or less severe phenotype of ADA deficiency.

1.1.6 Mouse Models of ADA Deficiency

There are no naturally occurring animal models of ADA deficiency, however two groups initially reported the generation of ADA deficient mice, which had severe metabolic abnormalities and died perinatally due to liver damage (Migchielsen *et al.*, 1995 & 1996; Wakamiya *et al.*, 1995). It was demonstrated that it was the lack of ADA which resulted in the perinatal death of these mice.

However, as a result of the perinatal death, the consequences of ADA deficiency on post-natal animals could not be assessed. Blackburn *et al.*, (1998 and 2000a & b) managed to rescue the ADA deficient mice by expressing the ADA transgene selectively in the placenta. Once born, these mice therefore lacked ADA expression, and were found to retain many features seen in ADA deficient humans, including immunodeficiency with combined T and B cell lymphopenia. They had smaller than usual spleens and thymuses, resulting in reduced lymphoid cell counts in these organs. The serum antibody levels decreased and B cell development was impaired (Aldrich *et al.*, 2003). The mice, like ADA deficient individuals, also had severe metabolic abnormalities and appeared to exhibit many non-lymphoid abnormalities.

They displayed bone and cartilage abnormalities of the ribs as well as renal abnormalities, attributed to the high levels of adenosine (Blackburn *et al.*, 2000b). These mice survived for 3 weeks before succumbing to respiratory failure. Respiratory problems have also been found in ADA deficient individuals; however they were usually thought to be as a result of bacterial or viral pneumonia. In most of these cases though, an infectious agent could not be isolated. The observations made in the ADA deficient mice may therefore suggest that the metabolic abnormalities may also be responsible for respiratory problems in ADA deficient patients. Blackburn *et al.* also showed that treating the mice with a low dose of enzyme replacement therapy rescued the mice and improved pulmonary deficiencies although the immune status did not appear to improve (Blackburn *et al.*, 2000b). It was found that a high-dose enzyme replacement therapy was required to decrease the metabolites in the spleen and thymus, increase the numbers of lymphocytes and thus improve immune function (Blackburn *et al.*, 2000a). It therefore appears that the pulmonary and immunological abnormalities are related to the severity of the metabolic disturbances, and these findings may be significant to the treatment of ADA deficient individuals.

Therefore, the generation of these ADA deficient mice is very significant as the molecular defects leading to the immunological and non-immunological symptoms can be studied in more detail and treatments can be assessed. However, one also needs to consider the fact that there are important differences in the requirement for ADA during development of the foetus in mice and humans, so any conclusions from these experiments need to be carefully assessed.

1.2 DIAGNOSIS AND TREATMENTS FOR ADENOSINE DEAMINASE DEFICIENCY

1.2.1 Diagnosis of ADA Deficiency

A common method of diagnosing ADA deficiency is by the measurement of ADA enzyme activity in erythrocytes, lymphocytes or fibroblasts. ADA deficiency is often diagnosed once the patient has been admitted to hospital due to severe persistent

infections, and has been found to have a B^-, T^-, NK^- SCID phenotype. In pedigrees with a family history, diagnosis can also be carried out pre-natally by measuring the enzyme activity in amniotic cells (Hirschhorn *et al.*, 1975; Hirschhorn, 1979b; Aitken *et al.*, 1980; Ziegler *et al.*, 1981) or chorionic villi (Aitken *et al.*, 1980; Dooley *et al.*, 1987). ADA deficient patients show very low or absent levels of ADA activity, whereas ADA carriers, who are generally healthy, have 5-80% of the normal ADA activity. ADA deficiency can also be diagnosed by measuring the amount of dATP in erythrocytes, which will be greatly increased in ADA deficient patients. Once ADA-SCID has been diagnosed by enzyme activity or dATP levels, it can be confirmed by DNA sequencing to reveal the mutation within the ADA gene, although this is not routinely undertaken in most centres.

1.2.2. Management of ADA Deficiency

At diagnosis, any infections must be specifically treated and the patient is given intravenous immunoglobulins (IVIG). The child should also be appropriately isolated (hence "the boy in the bubble" – Klug & Cummings, 1999) to avoid exposure to common childhood viruses, and live virus vaccines should be avoided. However, without restoration of immune function, ADA deficiency is fatal in the first 2 years of life usually due to infectious complications.

1.2.3 Bone Marrow Transplantation

There are few treatment options available for ADA-SCID. The preferred treatment is bone marrow transplantation if a fully matched donor is available, although this is the case only for approximately one-third of the patients. A matched transplant is usually successful, resulting in complete or partial immune reconstitution and a survival rate of greater than 90% is observed (Fischer *et al.*, 1990). This is thought to be due to the fact that no myeloablative conditioning is required prior to the transplant, as ADA deficient patients fail to produce B, T and NK cells. T cell engraftment usually occurs within a month, but humoral immunity recovers more slowly and is often not complete. Following BMT, metabolites in erythrocytes, plasma and urine are dramatically decreased (Chen *et al.*, 1978; reviewed by Hirschhorn, 1999). However, metabolites are still elevated, especially in plasma, when compared to normal, and SAH hydrolase activity remains low (Hirschhorn *et al.*, 1981 & reviewed by Hirschhorn, 1999). It has been shown that even in patients

who were immunologically normal 10 years following transplant, the deoxyadenosine and adenosine levels were still high in nonlymphoid tissues (Hirschhorn *et al.*, 1981), which may be important in regard to non-immunological symptoms of ADA deficient individuals.

If no matched donor is found, another option is to transplant mismatched marrow, which would be T cell depleted to minimise the risk of graft versus host disease (GVHD). However, as a result donor T cells do not return for 3 to 4 months and full T cell function may take longer to develop. It has also been found that B cell function remains abnormal, and consequently transplants from mismatched donors are less effective at restoring humoral immunity. Conditioning prior to a mismatched marrow transplant would usually need to be carried out to create 'more space' for the donor cells in the bone marrow. However, this leads to greater morbidity including complications such as renal and liver damage (Morgan *et al.*, 1991) and long-term problems such as infertility (Morgan *et al.*, 1991), dental defects (Cole *et al.*, 2000), and cognitive abnormalities (Phipps *et al.*, 1995). Transplantation without the use of conditioning would avoid the chemotherapy related complications, and has been found to increase survival rates. However, without the use of conditioning there is a greater risk of only partial immune reconstitution and a higher rate of rejection (Buckley *et al.*, 1999). The survival rate beyond three years for this type of transplantation is only in the region of 28-67% (O'Reilly *et al.*, 1989). It has also been noted that ADA deficient patients tend not to do as well as other SCID patients following this type of transplant. Therefore, due to greater morbidity and mortality associated with a mismatched transplant, this is normally not the treatment of choice.

1.2.4 Enzyme Replacement Therapy

An early form of enzyme replacement involved transfusions of irradiated red blood cells based on the observations that these cells have transport sites for both adenosine and deoxyadenosine and contain ADA (Polmar *et al.*, 1976). Although ADA activity levels increased and metabolite concentrations decreased, this response was usually found to be transient and inadequate (Polmar *et al.*, 1976; Cohen *et al.*, 1978; Schmalstieg *et al.*, 1978; Rubinstein *et al.*, 1979; Dyminski *et al.*, 1979; Hirschhorn *et al.*, 1980; Ziegler *et al.*, 1980; Hutton *et al.*, 1981; Davies *et al.*, 1982). It did

restore normal growth and development and prolonged survival in some of the patients, however any improvements in immunological function was short lived. This form of enzyme replacement therapy was therefore found to be ineffective and moreover carried the risk of transmitting infectious agents. As ADA has a half-life of only a few minutes, transfusions would need to be given constantly. This was not only impractical, but it also resulted in an increase in intracellular iron concentrations which lead to further toxic complications.

A more refined form of enzyme replacement therapy, polyethyleneglycol (PEG)-ADA therapy, was developed and patients who had survived with the RBC transfusions were quickly switched to this treatment. The covalent bonding of PEG to the ADA protein increased the half-life of the enzyme from minutes to days, making it more convenient to use. This treatment has been in use since 1987, it is thought to be safe and effective, and has seen good survival rates of nearly 80% (reviewed by Hershfield, 1995). One of the advantages of PEG-ADA is that unlike BMT it is not risky or invasive, and can therefore be offered even to seriously ill patients.

The treatment involves weekly or bi-weekly intra-muscular injections of PEG-ylated bovine ADA. It is an exogenous source of the enzyme, and relies on the fact that the metabolites are present in the plasma and body fluids and that the toxic metabolites such as dATP are in equilibrium with the precursor in the plasma so that when their concentrations are decreased in the plasma their intracellular concentrations are also decreased. When treating an ADA deficient patient, a rapid decrease in the toxic ADA metabolites dATP and dAdo is usually seen, resulting in levels that are lower than those seen following BMT, but are nevertheless slightly higher than normal (Hirschhorn *et al.*, 1980, 1981). As the metabolic defect is corrected by the exogenous ADA, SAHH activity increases to near normal, B cell numbers start to increase during the first month followed by T cells, and the lymphocyte counts then usually stabilise at levels slightly less than average. Although the lymphocyte numbers are near the lower end of the normal range, there is a good clinical protection from infection. PEG-ADA also results in restoration of normal growth and development and improves alertness. Several ADA-SCID patients have received PEG-ADA because they were considered too ill to undergo bone marrow

transplantation. However, the mortality rate among those patients was still less than 20%, which compares favourably to haploidentical BMT (Hershfield and Mitchell, 2001).

No formal studies exist on the outcome of patients treated with PEG-ADA, and the data presented is taken from reports from Hershfield. Following the first year of treatment, most patients remain lymphopenic, although specific antibody responses have been observed in 50% of the patients who no longer receive regular gammaglobulin treatment. It appears that humoral immunity improves to a greater extent in patients who respond to PEG-ADA than patients treated with BMT (Ochs *et al.*, 1992). Moreover, PEG-ADA unlike BMT is a systemic treatment, and in one study PEG-ADA administration resulted in complete normalisation of liver function tests in an ADA-SCID child who presented with persistent neonatal hepatitis for which no infectious cause could be found (reviewed by Hershfield & Mitchell, 2001). It is therefore possible that PEG-ADA, being a systemic treatment leading to a greater reduction of metabolites, may result in a greater improvement of non-immunological symptoms as compared with BMT.

However, the degree of immune reconstitution can be varied even though the PEG-ADA treatment improves the metabolic abnormalities. Although there have been no toxic or allergic reactions to PEG-ADA, approximately 50% of the patients develop antibodies against bovine ADA. This may reduce the effectiveness of the treatment, although this has been proven in only a few patients (Reviewed by Hirschhorn, 1999). Immune dysregulation also sometimes occurs as it does in BMT. Hershfield *et al.* (1993) found that two patients treated with PEG-ADA developed refractory immune haemolytic anaemia. One of these patients required immune suppression and as a result developed candida sepsis, leading to the death of the child. For the second patient, the PEG-ADA was withdrawn and a mismatched marrow transplant was attempted, though this was not successful as the patient died from complications related to the transplant. Another drawback with PEG-ADA is that it is not a curative treatment, and needs to be administered for the rest of the patient's life. Therefore, with the high cost of approximately $100,000 per year per patient, the PEG-ADA treatment is not always available to the child. Furthermore, the effect of PEG-ADA

administration long-term (>10 years) in terms of immune recovery and overall survival is not known.

1.2.5 Gene Therapy

For patients who do not have a matched donor, and for whom enzyme replacement is not effective or is not available, an attractive alternative is gene therapy. The first ever scientific article published mentioning the word "gene therapy" was in 1970, and speculated on the use of pseudoviruses containing therapeutic genes to transduce target cells (Osterman *et al.*, 1970). Since then hundreds of gene therapy trials have been carried out for several different diseases. Gene therapy involves the transfer of DNA or RNA into cells in order to correct or modify their functions. Modification of cell function has been attempted by increasing the immunogenicity of tumour cells (Nabel *et al.*, 1996), and correction of cell functions has also been attempted in diseases such as cystic fibrosis (Flotte *et al.*, 1996) (CFTR gene) and familial hypercholesterolemia (Wilson *et al.*, 1992) (LDL receptor).

1.2.5.1 Successful Gene Therapy for X-SCID

The first gene therapy trial to demonstrate therapeutic benefit was reported for X-SCID in the Paris trial in 2000 (Cavazzana-Calvo *et al.*, 2000). X-SCID, as previously mentioned, is a condition characterised by a lack of T and NK cells. These deficiencies occur as a result of a lack of the common gamma-chain, a subunit of several interleukin receptors (IL-2, IL-4, IL-7, IL-9, IL-11, IL-15 and IL-21). Consequently, cytokine signalling transmitting survival and proliferative signals to T and NK cells is affected resulting in compromised immune cell development and function. The role of the gamma chain in the survival and proliferation of the lymphoid cells means that its expression through gene therapy should confer a major selective advantage to the transduced cells (Soudais *et al.*, 2000). The Paris trial therefore utilised a gammaretroviral vector to introduce a functional gamma chain to autologous $CD34^+$ cells. A recombinant fibronectin fragment was used to facilitate co-localisation of the virus particle and target cell, thus increasing the gene transfer efficiency. 11 boys were treated in this trial, and all but one have shown good immune reconstitution (Hacein-Bey-Abina *et al.*, 2002). Immune reconstitution was rapid, with NK cells appearing at between 2 and 4 weeks post gene therapy, and the

number and distribution of T cells increasing rapidly. Humoral immunity was also partially restored. However, as a direct result of the gene therapy three patients developed leukaemia, one of whom died. This is further discussed in the general discussion.

Eight patients have also been treated in Great Ormond Street Hospital, London, using a similar protocol to the Paris study. However, in the London study, no FCS was used for cell culture and the gammaretrovirus was pseudotyped with the GALV envelope rather than the amphotropic envelope. These differences may have been responsible for the higher gene transfer efficiencies observed in B cells and myeloid cells in the London study. No adverse events have been observed to date, and seven of the eight patients have showed good immune recovery similar to those in the Paris study and have cleared viral infections. Prophylactic medication was withdrawn in two patients (Gaspar *et al.*, 2004). T cell responses to mitogenic and antigenic stimuli were normal and the T cell receptor repertoire was highly diverse following treatment. Where assessed, humoral activity, in terms of antibody production, was also restored. However one of the patients did not show immune reconstitution, possibly due to limited initiation of normal thymopoiesis as a result of older age at treatment, also observed in the Paris study (Howe *et al.*, 2003; Thrasher *et al.*, 2005). Therefore, therapeutic potential of gene therapy was first demonstrated for X-SCID.

1.2.5.2 Gene Therapy for ADA-SCID

The first inherited disease for which therapeutic gene therapy was undertaken was ADA deficiency in the early 1990's (Blease, 1992; Bordignon *et al.*, 1995; Blease *et al.*, 1995; Kohn *et al.*, 1995; Hoeben *et al.*, 1992; Hoogerbrugge *et al.*, 1992), and since then 24 ADA-SCID patients have been treated. ADA appeared to be a good candidate for gene therapy for several reasons, firstly because it is caused by a single gene defect. The gene was identified in 1983 (Valerio *et al.*, 1983; Wiginton *et al.*, 1983; Orkin *et al.*, 1983), which has allowed a great deal of research to be carried out on its function and regulation. Importantly, since ADA is a housekeeping enzyme, its expression does not need to be tightly regulated. In fact, individuals with as little as 5% of and as much as 50 times more than normal ADA activity have been found to have functioning immune systems. Furthermore, murine models have shown that

gene corrected lymphoid cells have a survival advantage over the non-transduced cells (Ferrari *et al.*, 1991). Clinical observations have also been encouraging, as bone marrow transplantations have similarly shown that ADA containing cells have a selective advantage over ADA deficient cells (Parkman *et al.*, 1975; Tjonnfjord *et al.*, 1994). Another remarkable case saw an ADA deficient child improve clinically without any therapy. This was found to be due to a reversion of the mutation in a T lymphoid progenitor which then repopulated the entire T cell population, again showing evidence of selective advantage of ADA^+ cells (Hirschhorn *et al.*, 1994; Hirschhorn *et al.*, 1996). Therefore, for these reasons, gene therapy for ADA deficiency was considered to have good chances of success, and clinical trials were therefore initiated following further encouraging results in preclinical studies.

Preclinical studies to ADA gene therapy were carried out in mice in the mid-1980's, subsequent to reports that cultured human B and T cells transduced with an ADA retrovirus showed reduced sensitivity to 2-deoxyadenosine toxicity (Kantoff *et al.*, 1986). The murine study involved *ex vivo* gene transfer of the human ADA gene into haematopoietic stem cells (HSCs), which were reinfused into irradiated mice (Lim *et al.*, 1987). Protein analysis showed that circulating haematopoietic cells that had been recovered were expressing the human ADA. Based on these preclinical observations, a similar protocol was suggested for use in ADA deficient patients. The proposed protocol involved the harvest of autologous bone marrow and transduction of the bone marrow cells *ex vivo* using a murine gammaretrovirus carrying the ADA cDNA prior to reinfusion of the transduced cells. This type of protocol was used in pre-clinical trials in rhesus monkeys (Van Beusechem *et al.*, 1992), which showed that the retroviral transduced HSCs were capable of repopulating the haematopoietic compartment of irradiated recipients. Although the level of transgene expression was low, it was detectable in several haematopoietic cells at various times including at more than one year post gene therapy, and the therapy proved to be safe. Therefore, all preclinical trials supported the feasibility of gene therapy for ADA deficiency in humans.

Human gene therapy trials that have been carried out for ADA deficiency to date are listed in Table 1.2. Some of these trials have encountered difficulties, mainly relating to transduction of stem cells. This is partly due to the low numbers of stem cells in

the bone marrow (less than 0.1% of bone marrow mononuclear cells (MNC)), and the mainly quiescent status of the cells resulting in low gene transfer efficiency. Cytokine inductions of these stem cells are therefore required to stimulate cell division and thus allow gammaretroviral transduction, however this usually also leads to stem cell differentiation, reducing their multipotential nature. These problems have been addressed in studies which have analysed vector design (Armentano *et al.*, 1987; Hantzopoulos *et al.*, 1989; Hock *et al.*, 1989; Van Beusechem *et al.*, 1990) and cytokine cocktails used to induce proliferation without differentiation (Bodine *et al.*, 1989; Fletcher *et al.*, 1991).

Study	**Gamma-retroviral Vector**	**Envelope**	**Cell Type**	**Number of Patients**
Blease, NIH, 1990	LASN	A	PBL	2
Bordignon, Milan, 1992	DCAm, DCAl	A	BM $CD34^+$ & PBL	2
Kohn, L.A., 1993	LASN	A	UCB $CD34^+$	3
Hoogerbrugge, Netherlands, 1993	LgAL	A	BM $CD34^+$	3
Onodera, Japan, 1995	LASN	A	PBL	1
Aiuti, Milan, 2001	GIADA1	A	BM $CD34^+$	6
NIH, 2001*	N/A	N/A	N/A	4
Sakiyama, Japan, 2005	GCsapM-ADA	N/A	BM $CD34^+$	2
Gaspar, London, 2003	SFada/W	G	BM $CD34^+$	1

Table 1.2. Gene therapy trials carried out for ADA deficiency.

Abbreviations: A, amphotropic; PBL, peripheral blood lymphocytes; BM, bone marrow; UCB, umbilical cord blood; N/A, information not available; G, GALV.

* Details have not been published to date.

1.2.5.3 ADA Gene Therapy – Study by Blease *et al.*

As transduction conditions of HSCs had not been fully determined at the time, the first human gene therapy trial for ADA deficiency carried out at the NIH (Blease, 1992; Blease *et al.*, 1995) utilised mature T cell lymphocytes. In this trial, two patients were treated, both of whom received monthly or bi-monthly injections of transduced T cells following stimulation using anti-CD3 antibody and IL-2. The vector used was the LASN (LTR-ADA-SV40-NeoR) gammaretroviral vector with an amphotropic envelope, and ADA expression was driven by the Moloney murine leukaemia virus (MoMLV) LTR. Crucially, this trial demonstrated that there was no toxicity related to the gene therapy. Follow-up studies also showed that although there was no great therapeutic effect, the transgene still persisted 10 years following the treatment of one of the patients. Approximately 30% of the T cells were transduced in that patient, whereas the second patient had less than 1% transduced T cells (Muul *et al.*, 2003). This difference in transduced cells is possibly due to the great difference in original transduction efficiencies of the two patients' cells (1-10% in the first patient and 0.1-1% in the second patient), or due to an immune response in the second patient to foetal calf serum (Tuschong *et al.*, 2002) and a viral envelope protein (Muul *et al.*, 2003). Both patients did have improved antibody titres and improved delayed hypersensitivity skin tests following gene therapy. However, reasons as to why gene therapy did not entirely correct the immune system are thought to be related to low transduction efficiency. Moreover, the patients were receiving PEG-ADA before the trial and at a reduced dose after the trial, and it is now believed that maintaining the patients on PEG-ADA removed or blunted the possible selective advantage of the ADA^+ cells. Nevertheless, questions remained regarding the persistence of the transduced T cells without exogenous ADA and whether T cells alone would be sufficient for patient protection. Hence it was important to carry out further trials using stem cells that should provide the patient with a more varied repertoire of transduced immune cells. The possibility of discontinuing PEG-ADA treatments in gene-therapy candidates was also considered in order to enhance any selective advantage of transduced cells, thus allowing a better reconstitution of immune function.

1.2.5.4 ADA Gene Therapy – Study by Bordignon *et al.*

The first gene therapy trial using HSCs was reported by Bordignon *et al.* in 1995. It involved the *ex vivo* transduction of both bone marrow derived HSCs and peripheral blood derived mature T lymphocytes using two different gammaretroviral vectors in order to facilitate the identification of the origin of recovered transduced cells. The patients received further injections of transduced T cells over time. Shortly after gene therapy circulating transduced T lymphocytes were detected originating from the peripheral blood derived T lymphocytes. However gradually more transduced cells could be identified originating from the transduced HSCs. Clinical improvement was observed as height and weight of both patients normalised, however, overall improvement could not be attributed to gene therapy because the patients were also receiving PEG-ADA. Importantly, analysis for recombinant virus was consistently negative.

1.2.5.5 ADA Gene Therapy – Study by Kohn *et al.*

Kohn *et al.* carried out another gene therapy trial utilising HSCs. They transduced HSCs from umbilical cord blood of three pre-natally diagnosed ADA deficient patients using the NIH LASN vector (Kohn *et al.*, 1995). These children received PEG-ADA from the first week of their lives. This study gave rise to several interesting observations regarding the possibilities for gene therapy, but also its limitations. It was found that even without cytoablative therapy, approximately 20% of granulocyte-macrophage colony-forming unit (CFU-GM) progenitor cells from UCB contained the transgene, although this decreased to 1% one year later in bone marrow cells. This treatment was announced a success as the children developed a functional immune system, and the immunoglobulin replacement therapy was removed. Two years following their gene therapy treatment, the PEG-ADA was gradually decreased. As the PEG-ADA was lowered, there was a marked decrease in circulating T cells but a 100-fold increase was seen in transduced T cells, suggesting a selective survival advantage of the transduced cells. Four years post gene therapy, PEG-ADA treatment was discontinued in one patient for two months as 10% of his T cells were transduced and it was hoped that the selective advantage introduced to these cells would result in an increase in their numbers. However, as the PEG-ADA was removed, the plasma ADA levels decreased, the dAdo increased to the high

levels seen prior to PEG-ADA therapy, and the S-adenosyl homocysteine hydrolase activity therefore decreased (Kohn *et al.*, 1998). Moreover the absolute numbers of NK and B cells decreased to less than 25% compared with when he was receiving PEG-ADA. Although the number of T cells decreased, the frequency of transduced T cells increased to 30%, which is consistent with the selective advantage seen following bone marrow transplantations. However, the patient developed oral thrush, an upper respiratory infection and weight loss two months following discontinuation of PEG-ADA and was therefore reinstated on therapeutic doses of PEG-ADA. Upon reintroduction of exogenous ADA the patient immediately showed signs of immunological improvement with a decrease in dAdo, increase in B and NK cells and a reappearance of antigen-specific T lymphocytes. It therefore appeared that the transduced cells were not capable of sustaining the immune system without the presence of exogenous ADA.

1.2.5.6 ADA Gene Therapy – Study by Hoogerbrugge *et al.*

Another trial using HSCs was carried out by Hoogerbrugge *et al.*, who utilised bone marrow derived HSCs to treat three patients (Hoogerbrugge *et al.*, 1992 & 1996). However, they did not achieve a good transduction efficiency, and as a result no transduced cells were detected long term. The lack of transduced cells long term was also attributed to the lack of myeloablation and the continuation of the PEG-ADA treatment removing the selective advantage of ADA^+ cells. Therefore, no specific conclusions could be drawn from this study.

1.2.5.7 ADA Gene Therapy – Study by Onodera *et al.*

Onodera *et al.* treated one ADA deficient patient, who has showed some immune reconstitution (Onodera *et al.*, 1998). The NIH LASN vector was used to transduce T lymphocytes, and the patient was given ten infusions of the autologous cells over an 18-month period. One year after the last infusion, circulating PBLs remained 10-20% transduced, resulting in ADA activity levels in T cells similar to ADA carriers. The T-lymphocyte counts increased and his immune function was improved. However, at the time of publication, there had only been a period of one year post gene therapy, hence follow-up data will be needed to determine the success of this trial. This group

has since treated two more patients in the absence of PEG-ADA and no conditioning (Sakiyama *et al.*, 2005).

1.2.5.8 ADA Gene Therapy – Study by Bordignon & Aiuti

Aiuti *et al.* (2002b) showed that withdrawal of PEG-ADA can lead to improved immune reconstitution, contrary to the results of Kohn *et al.* in 1998. One of the patients in the study by Bordignon (Bordignon *et al.*, 1995) had a frequency of transduced lymphocytes of 1-3% two years post gene therapy. The PEG-ADA, administered throughout the gene therapy study was found not to support T-lymphopoiesis, as the patient remained lymphopenic, had low response to antigens and reduced thymic function. It was therefore decided to discontinue PEG-ADA gradually to reintroduce the selective advantage of the transduced cells, along with further infusions of transduced PBLs. Following discontinuation of PEG-ADA, the proportion of transduced T cells increased, supporting the notion of selective advantage of ADA^+ cells. The absolute T cell counts also increased, along with an increase in PBL ADA activity, allowing for complete restoration of T cell functions. Following an infection, the PEG-ADA was reinstated briefly and once the infection was cleared, PEG-ADA was again discontinued. Following the second discontinuation, the levels of deoxyribonucleotides were found to decrease in the erythroid cells, suggesting that a certain number of transduced lymphocytes may be required for detoxification. Hence, these observations seem to imply that a selective advantage of transduced cells is important for the success of gene therapy for adenosine deaminase deficiency. Reasons as to why PEG-ADA withdrawal was successful in this study contrary to the findings of Kohn *et al.*, may be related to the different target cells or the difference in number of transduced cells infused, or simply due to different responses to treatment seen in ADA deficient individuals.

1.2.5.9 ADA Gene Therapy – Study by Aiuti *et al.*

A recent study using a modified protocol has had even more success. Aiuti *et al.* (2002a) used transduced autologous $CD34^+$ cells to treat two patients, but unlike previous studies these patients did not commence on PEG-ADA due to socio-economic reasons. The patients were also given a mild nonmyeloablative

conditioning regimen, in order to create 'more space' for the transduced haematopoietic stem cells. Levels of engraftment of genetically corrected cells were therefore improved. The proportions of vector containing T cells were found to peak at 70% 11 months following gene therapy for Patient 1, and at 100% at day 240 for Patient 2. In Patient 1, 5-20% of granulocytes, monocytes, megakaryocytes and erythroid cells were found to contain the transgene a year following therapy. Gene therapy therefore resulted in increased lymphocyte counts, normalised serum immunoglobulin levels and improved immune systems concomitant with lower toxic ADA substrates and increased ADA activity levels. Both patients are now at home and are clinically well, showing normal growth and development. Interestingly, patient 1 has showed a better immune reconstitution than patient 2. There are three reasons thought to be important in this difference of response. Firstly, patient 1 received a log higher in number of transduced $CD34^+$ cells than patient 2. Secondly, patient 1 was only 7 months old whereas patient 2 was 2.5 years old, and this could be important for the level of HSC engraftment. Another important factor could be the degree of conditioning in each patient as a result of different routes of administration. It therefore appears that there are several factors in the gene therapy protocol crucial to its success, including the number of infused transduced cells, the level of conditioning achieved and the age of the patient at treatment. Following the successful treatment of these two patients, 3 further patients have been treated with similarly encouraging results (Aiuti *et al.*, 2005).

1.2.6 Limitations of Current Treatments for ADA Deficiency

It has been demonstrated that ADA deficiency is a multi-organ disease, hence requiring a systemic treatment. Bone marrow transplantations have shown improved immunological function, however it is not a systemic treatment and the metabolites do remain above the normal range. PEG-ADA is a more systemic treatment, resulting in elimination of toxic metabolites. However, it is not a good long-term treatment as the effectiveness may decline with time, and the treatment becomes more expensive as the child grows and requires higher doses of enzyme. Therefore there is a need to develop a more long-term and systemic treatment for ADA deficiency.

Correction of mature T cells or haematopoietic stem cells currently performed in gene therapy trials may not be sufficient in the case of ADA deficiency, as it has been shown to be a systemic disease. Hence the use of mesenchymal stem cells (MSCs) which are believed to be multipotential (discussed below) in combination with HSCs may be an alternative to the use of HSCs alone as it may offer a more systemic treatment. It was seen in the Italian study that gene therapy for ADA deficiency without the use of PEG-ADA results in a greater proportion of transduced circulating cells due to a selective advantage of these corrected cells. Hence patients already receiving PEG-ADA should probably have this discontinued prior to gene therapy. Mild conditioning also appears to increase the levels of engraftment of the transduced cells as it makes space in the bone marrow. Therefore gene therapy using lentiviral vectors to transduce HSCs and MSCs, the removal of PEG-ADA and the conditioning of the patient prior to gene therapy may offer a good treatment for ADA deficiency.

1.3 COMPONENTS REQUIRED FOR GENE THERAPY OF ADA DEFICIENCY

1.3.1 Gene Delivery Vehicles

As indicated in Table 1.2, all ADA-SCID gene therapy trials to date have utilised gammaretroviral vectors as gene delivery vehicles. Retroviral vectors have several advantages, including a large packaging capacity of up to 10 kilobases, efficient integration machinery, long-term expression of the transgene, and the absence of vector-induced cellular immune response as they do not encode viral specific proteins. Therefore it is thought that gammaretroviral or lentiviral vectors are the most appropriate gene delivery vehicles for diseases such as ADA deficiency where long-term gene expression and a lack of immune response are required.

Other viral vectors developed for gene therapy include lentiviruses, adenovirus, adeno-associated virus (AAV) and Herpes Simplex Virus (HSV). Lentiviruses are currently being developed and are further discussed in section 1.3.1.2. Adenoviral vectors are not appropriate to treat ADA deficiency as they show high

immunogenicity and do not integrate, which would mean that the transgene would not be expressed long-term. Furthermore, cell specific targeting with adenovirus is difficult to achieve as it does not have an envelope to attach cell specific ligands (Gilgenkrantz *et al.*, 1995; Yang & Wilson, 1995). AAV has been found to be inefficient at transducing primary cells, making it difficult to use for HSC transfer (Halbert *et al.*, 1995). HSV has been evaluated for gene therapy for neuronal diseases such as Parkinson's Disease, as it shows a long-lived asymptomatic stage in sensory neurons. However, its safety is of some concern as the wild-type virus has been shown to replicate lytically in human brain (reviewed by Latchman, 1994). Non-viral gene therapy is also under current development in order to try to improve the safety of the vector and to reduce the inflammatory response sometimes seen with viral vectors. However, non-viral vectors are still much less efficient than viral vectors, and the general goal of the development of non-viral vectors is to add features to DNA to mimic those functions that viruses already perform well. Therefore, non-viral vectors were not considered for gene therapy of ADA deficiency.

1.3.1.1 Gammaretroviral Vectors as Gene Delivery Vehicles

As a delivery tool, there are several important features of the viral vector. These include efficiency of transduction, the ability to target the cell types of interest, the expression of the transgene and the safety of the viral vector. A significant feature of gammaretroviral vectors for gene therapy is their ability to integrate into the genomic DNA of the host cell. Thus they can be used to introduce and permanently express genes in host cells, making them ideal delivery vectors to provide permanent correction of genetic diseases affecting the haematopoietic system (Karlsson, 1991).

The retroviral particle is composed of two single stranded RNA molecules, which are surrounded by a capsid and an envelope. The viral genome is composed of a 5' and a 3' LTR which contain promoter and enhancer elements, where the U3 region of the 5' LTR drives proviral transcription. Between the LTRs, the retrovirus contains the coding regions *gag-pol-env* in this order. *Gag* encodes structural proteins of the capsid, *pol* encodes the polymerase and integrase proteins, and *env* encodes the viral envelope. An important sequence element is the packaging sequence (ψ) which has been shown to play a role in assembling genomic RNA into virions. Attachment

(Att) sites within the LTRs have been found to be important as these are the sites where the integrase cleaves and binds the proviral ends to the host genomic DNA (reviewed by Luciw & Leung, 1992).

The viral life cycle has been extensively studied and involves the attachment to the host cell via receptors, internalisation, uncoating, reverse transcription, transportation to the nucleus and integration of the cDNA into the host cell genomic DNA to generate an integrated provirus (Figure 1.5). The integrated provirus is replicated during the cell cycle and passed on to daughter cells. The retroviral coding sequences are then expressed by the host cell transcriptional machinery. The newly synthesised viral proteins and RNAs are assembled to form new viral particles that bud out of the host cell, taking with them part of the cell membrane as a viral envelope (reviewed by Coffin, 1996). Reverse transcription is a complex mechanism by which the viral genomic RNA is transcribed into DNA and it is at this point that many mutations are introduced, especially important in HIV virulence. During reverse transcription a copy of the 3' LTR is transferred onto the 5' LTR. This strand-transfer step is exploited in the safety design feature of SIN vectors described in section 6.1.

Preferred sites of integration for gammaretroviral vectors have been particularly intensively studied following the leukaemia cases in three children in the Paris gene therapy trial. It was thought that integration was random, but it has now been revealed that gammaretroviruses have a strong preference for gene vicinity (Wu *et al.*, 2003), which is discussed in more detail in section 8.2.1.

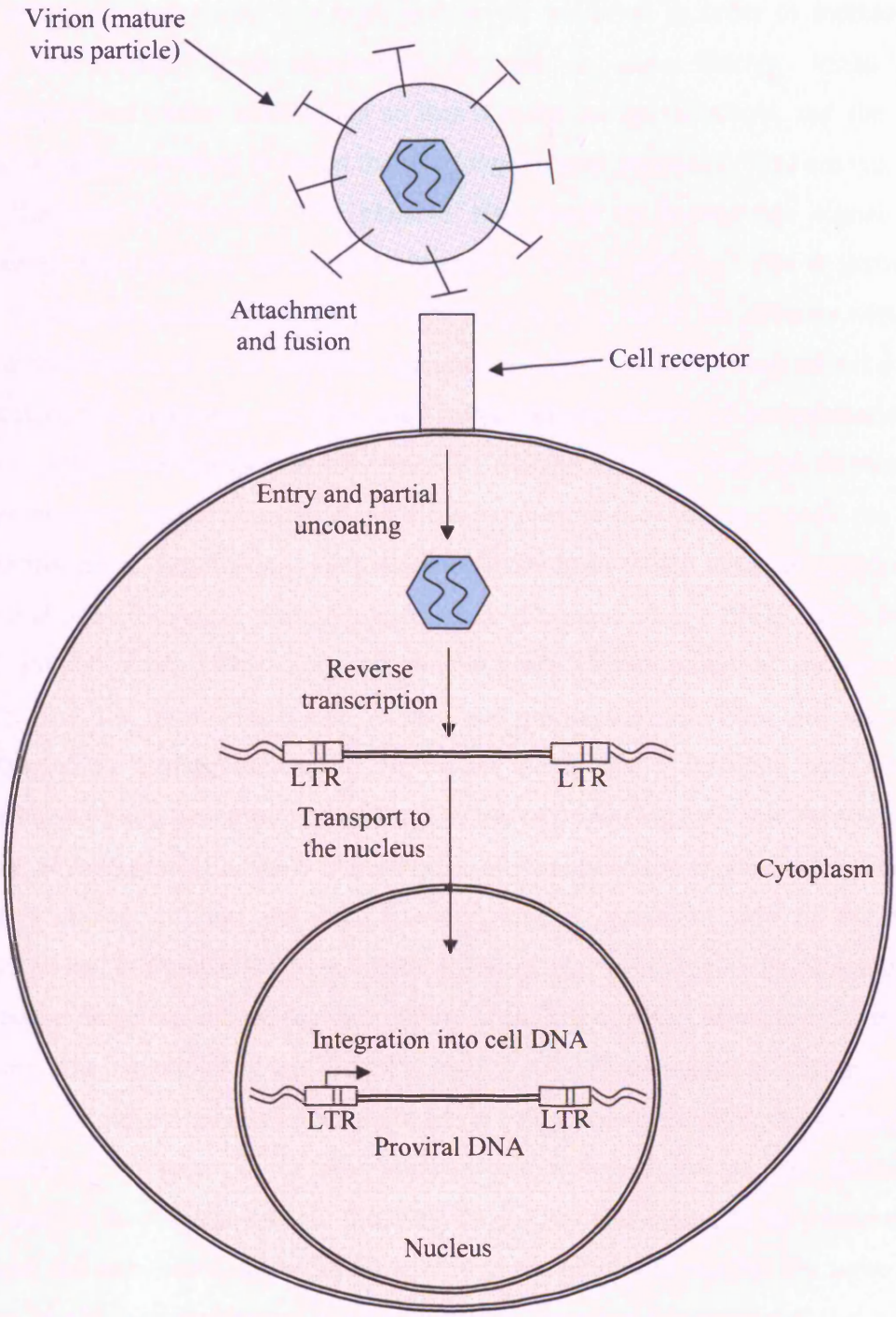


Figure 1.5. Wild type retroviral lifecycle. Attachment of the virion is mediated by a receptor. The virus is then internalised, the RNA is reverse transcribed, and the DNA is integrated into the host cell's genomic DNA where it becomes a provirus. (Adapted from Luciw & Leung, 1992).

The wild type viral vector has been extensively modified in order to increase its safety and comply with regulations for use in gene therapy trials. The gammaretroviral vector is designed so that it is replication defective, and the viral coding sequences present between the two long terminal repeats (LTRs) are replaced with the gene of interest. The plasmid also needs to incorporate signals for packaging, reverse transcription and integration. A packaging cell line is generally used to generate gammaretroviral particles (Figure 1.6). All of the proteins required for the production of virus particles (gag-pol and env) are stably maintained in the genome of the packaging cell line. This minimises the chance of recombination to produce replication competent retrovirus (RCR), and the virus particles themselves cannot produce further generations of virus particles as they do not encode the gag, pol or env genes. Replication competent MLV has been shown to cause lymphomas in primates used in gene therapy experiments (Donahue *et al.*, 1992; Vanin *et al.*, 1994; Purcell *et al.*, 1996). Thus, all clinical grade vectors produced are tested for RCRs, and the gammaretroviral vectors and packaging cell lines are carefully constructed to avoid overlapping sequences which could facilitate homologous recombination and generation of RCRs. The use of packaging cell lines therefore has several advantages including a minimal risk of recombination to generate infectious virus, a choice of viral envelope pseudotyping, a consistent titre of the virus generated and is amenability to industrial scale-up. However, due to the design of the replication defective vector, the multiplicity of infection (MOI) needs to be carefully selected. The separation of env from the viral vector expressing the transgene means that once the retrovirus has infected the target cell, a new generation virus cannot be generated as no gag, pol or env proteins are expressed. As no env protein is expressed in the transduced cell, there are no env proteins bound to the receptors of the host cell membrane, allowing for several viral particles to reinfect the same cell. Hence, if a high concentration of virus is used to transduce the target cell (i.e. a high MOI) several viral particles may integrate into the host cell genome and thus increase the risk of insertional mutagenesis. It is therefore important to balance the need for a high transduction efficiency, which usually results in a high transgene expression, and the risk of leukaemogenesis.

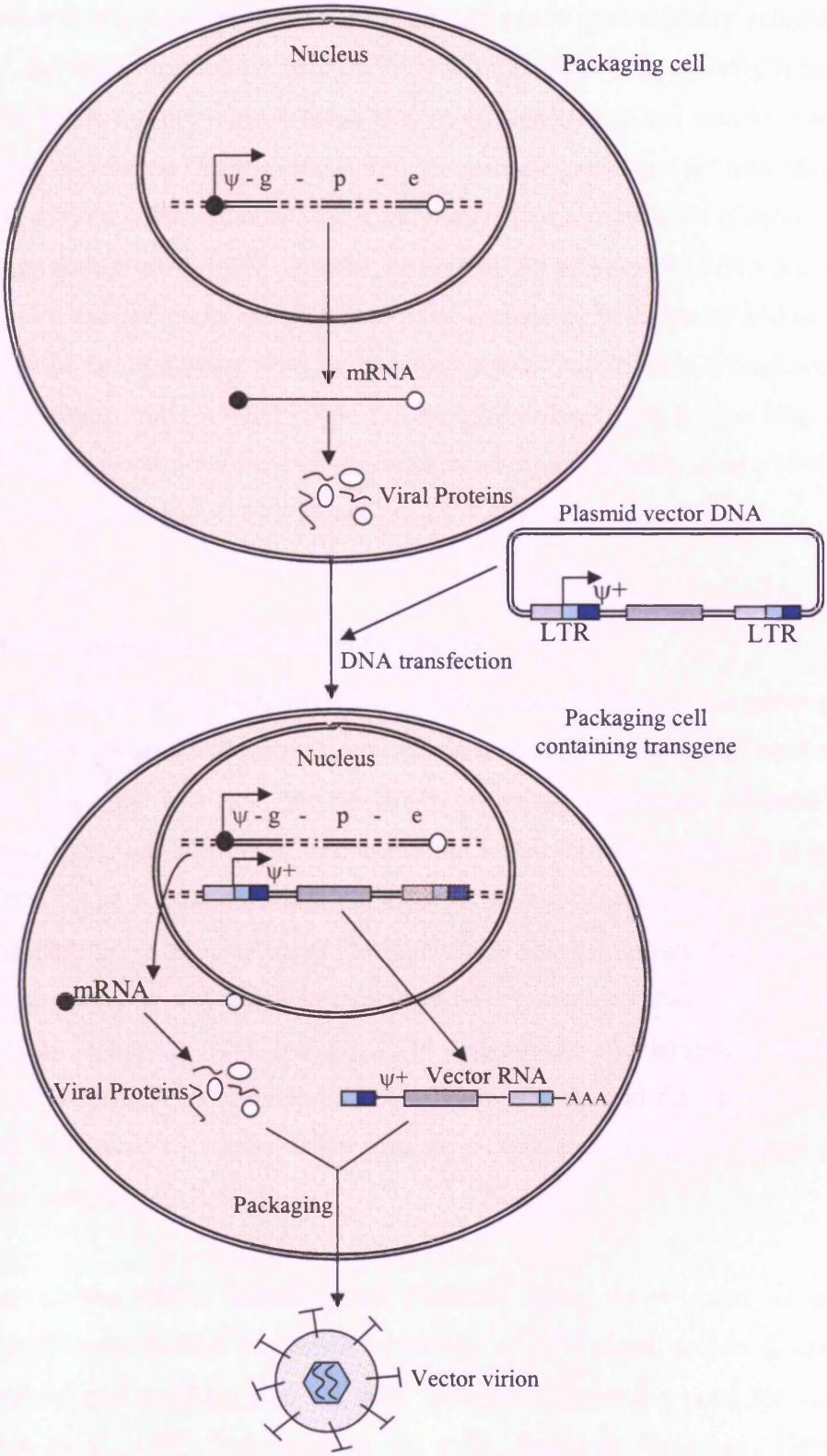


Figure 1.6. Principles of a gammaretroviral packaging cell line. The packaging cell line provides the genes required for virus particle formation (gag, pol and env contained separately within the genome) which have been deleted from the vector. The packaging cell line is transfected with the plasmid vector DNA containing the gene of interest and the packaging signal ψ. Transcripts of the transfected plasmid are packaged into virions. These viral particles contain all functions required for one round of transduction and integration. (Adapted from Luciw & Leung, 1992).

Although gammaretroviruses clearly have many advantages as gene delivery vehicles in ADA-SCID, the main limitation is their inability to infect non-dividing cells. It has been shown that preintegration complexes (PICs) of gammaretroviruses cannot cross an intact nuclear membrane (Roe *et al.*, 1993). As a result gene transfer into slow dividing or non-dividing cells, such as HSCs and mesenchymal stem cells (Gothot *et al.*, 1998; Conget & Minguell, 1999), is quite inefficient. As most of the HSCs are in the G0/G1 stage of the cell cycle, the cells need to be stimulated to divide by addition of specific cytokine combinations. This extended *ex vivo* culture of HSCs improves transduction efficiency but inevitably also results in differentiation of the HSCs, reducing their multipotential nature and engraftment potential (Bhatia *et al.*, 1997; Gothot *et al.*, 1998, Rebel *et al.*, 1999; Demaison *et al.*, 2000).

1.3.1.2 Lentiviral Vectors as Gene Delivery Vehicles

Lentiviruses are another family of retroviruses which have a more complex genome and replication cycle. Whereas the gammaretrovirus described in the previous section expresses only gag, pol and env, the lentivirus expresses additional accessory proteins including Tat, Rev, Nef, Vif, Vpr, and Vpu, whose functions are still to be fully elucidated. Tat is a transcriptional activator, Rev regulates transport of full-length or single-spliced viral RNA from the nucleus to the cytoplasm, Nef down-regulates the CD4 receptor and plays an important role in viral pathogenesis, Vif is important in viral infectivity, Vpu initiates CD4 degradation and enhances virion release, and Vpr is thought to be important in cell cycle arrest and fidelity of viral DNA synthesis (reviewed by Luciw, 1996; Mansky, 1996; reviewed by Frankel & Young, 1998; Mansky *et al.*, 2000).

Vectors based on the HIV-1 lentivirus are currently being investigated as an improved gene delivery system. The main advantage of HIV-based vectors is that they can transduce and integrate into the cells' genome without the need for cell division (Lewis *et al.*, 1992; Bukrinsky *et al.*, 1993; Lewis & Emerman, 1994; Naldini *et al.*, 1996a). Whereas gammaretroviruses rely on the breakdown of the nuclear membrane during mitosis (Miller *et al.*, 1990; Roe *et al.*, 1993), lentiviruses have evolved properties relying on the use of a nuclear import pathway enabling the viral DNA to cross the nuclear membrane of the infected cell. Several different

lentiviral proteins that control nuclear entry have been identified although none of them appear to be dominant (Gallay *et al.*, 1995a & b, 1996, 1997). Three nuclear localisation motifs have been found in HIV integrase, however the exact localisation of these sequences are still uncertain (Gallay *et al.*, 1997). One of the factors thought to be required for nuclear entry is the central flap sequence, the central polypurine tract (cPPT) (Follenzi *et al.*, 2000). This DNA flap (so named because during reverse transcription, a central strand displacement following central initiation and termination of plus strand synthesis creates an stable plus strand overlap at the centre of the linear DNA molecule) has been shown to be involved in viral genome nuclear transport (Zennou *et al.*, 2000), although the mechanism by which it enhances viral genome uptake into the nucleus has not yet been fully elucidated. It has been speculated that the central DNA flap may directly interact with components of the nuclear pore, or may be involved in the maturation of the HIV capsids into pre-integration complexes. Zennou *et al.* found that the presence of the DNA flap increased transduction in HeLa cells by more than ten-fold. The inclusion of the cPPT element has also been shown to improve gene transfer into haematopoietic stem cells and the consequent gene expression (Sirven *et al.*, 2000; Demaison *et al.*, 2002; Manganini *et al.*, 2002). In fact, Sirven *et al.* showed that approximately 40% of $CD34^+$ cells were transduced with lentivirus containing the cPPT element during the short culture period of 24 hours in the presence of cytokines. Two recent studies have shown that the lentivirus does not rely on the cPPT element for nuclear entry, however at a low MOI it appears that the flap gives the lentivirus an advantage for transduction of quiescent cells (Dvorin *et al.*, 2002; Limon *et al.*, 2002). Dvorin *et al.* also discovered that a valine residue at position 165 in the viral integrase was more important for nuclear translocation of viral nucleic acids than the cPPT element. This demonstrates that the process of nuclear translocation of lentiviral vectors is still not fully elucidated and that more studies are required to clarify this process.

As the lentiviral vector is based on the structure of HIV (usually HIV-1), the safety of the lentiviral vector has been of concern. In order to be considered for clinical applications, lentiviral vectors must comply with strict safety standards. This has been addressed by several groups and multiple alterations have been made to the vector in order to improve its safety. The modifications have occurred in a step-wise manner over time, and the different viral vectors are categorised into "generations".

In each of the different generations of lentiviral vectors, genes are deleted and split onto different vectors and sequence overlap is minimised to reduce the possibility of the generation of replication competent lentiviruses (RCL). The first generation design is generated from three plasmids: a *gag-pol* packaging plasmid, comprised of all of the HIV-1 proteins except the envelope, the transgene plasmid and a VSV-G envelope plasmid (Naldini *et al.*, 1996b). This split genome system meant that multiple rearrangements and recombination events would be required to form a "wild-type" lentivirus. The second generation lentivirus is also generated from three separate plasmids (Figure 1.7). However, this vector has alterations in the gag-pol packaging construct, where the accessory genes *vpr*, *vif*, *vpu* and *nef* have been deleted, as these are not crucial for viral growth *in vitro*, but are vital for *in vivo* replication and pathogenesis. Thus only the two regulatory elements tat and rev are retained, and the 3' LTR is replaced with a poly-A site. Hence five out of nine genes of the parental virus have been deleted, and this multiply attenuated lentivirus has been proven to retain the ability to transduce quiescent cells (Zufferey *et al.*, 1997). Thus, there are three separate expression cassettes which have very little sequence overlap in order to minimise the chance of homologous recombination to generate a replication competent lentivirus. In the third generation vector, tat and rev have also been deleted from the gag-pol packaging plasmid (Dull *et al.*, 1998). However, Rev positively affects the nuclear export of the unspliced gag-pol mRNA as well as the transfer vector genomic RNA. Therefore, as rev is still required, this is supplied by a fourth plasmid in order to minimise the chance of recombination events to generate a RCL. Tat, which binds to TAR in the transfer vector, is a crucial transactivator for lentiviral replication. However, it can be removed if a different promoter is used in the transfer vector. The deletion of Tat therefore improves the safety of the lentivirus as the infectivity is reduced. An additional safety feature utilised in most lentiviral vectors is the self-inactivating (SIN) system described in section 6.1.

Therapeutic efficacy of lentiviral vectors has been shown in murine and primate models for different diseases including mucopolysaccharidosis type VII (Bosch *et al.*, 2000), metachromatic leukodystrophy (Consiglio *et al.*, 2001) and Parkinson's disease (Kordower *et al.*, 2000). The first clinical trial in humans involving a lentiviral vector was recently initiated (MacGregor, 2001), which involved the expression of an anti-sense to the HIV envelope gene for the treatment of AIDS.

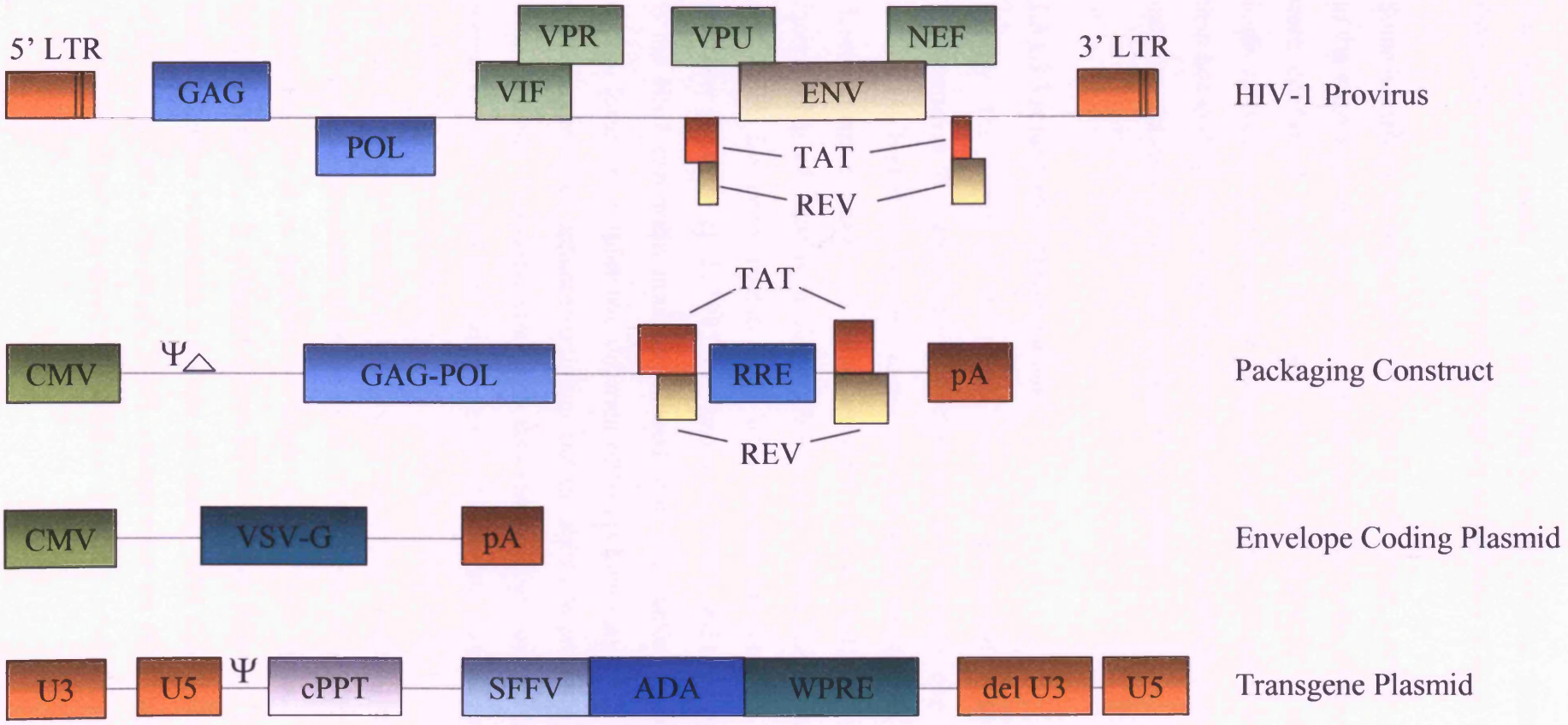


Figure 1.7. Schematic diagram comparing the HIV provirus with the three plasmids used to generate the second generation lentiviral construct (not to scale). The elements of the HIV provirus required for one round of transduction have been placed on three separate plasmids in order to minimize the chance of recombination to produce a wild type virus. The lentivirus is pseudotyped with the VSV-G envelope which has a broad host range and results in a virus of high stability. The cPPT element has been included for efficient transduction. The internal promoter SFFV drives the expression of the ADA transgene, whose expression levels are increased by the WPRE. The U3 region of the 3' LTR has been deleted to generate a safer SIN vector. (Adapted from Naldini *et al.*, 1996a).

The long-term results of this and other lentiviral clinical trials will need to be thoroughly evaluated to determine the safety and efficacy of these viral vectors.

Some limitations with the use of lentiviruses still persist. Lentiviruses may be subject to the same silencing mechanisms as gamma-retroviruses, although this has not yet been confirmed. It has also been shown that, similarly to gammaretroviruses, lentiviral vectors preferentially integrate into transcriptionally active genes rather than non-coding regions of chromatin (Schroder *et al.*, 2002). It was determined that most integrations (67%) were localised within genes.

1.3.1.3 Vector Safety Developments

Clearly, the safety of the viral vector is paramount, hence several safety improvements to vectors have been done and are currently ongoing. This includes minimising homology between vector and packaging constructs (Dull *et al.*, 1998), developments of new and improved SIN vectors (Zufferey *et al.*, 1998) and incorporation of flanking insulators (Emery *et al.*, 2000; Ramezani *et al.*, 2003; Li *et al.*, 2005). Insulators operate only when located between the cis effectors and promoter (Udvardy *et al.*, 1985; Prioleau *et al.*, 1999), and the most studied insulator is the HS-4 chromatin insulator derived from the chicken β-globin locus control region. Insulators display two different activities both very useful for gene therapy: the ability to block enhancer activities and the ability to protect expression cassettes from silencing. Transgene expression thus remains high, and the frequency of vector mediated activation of host genes can be reduced, thus decreasing the rate of vector mediated genotoxicity (Emery *et al.*, 2000; Ramezani *et al.*, 2003; Li *et al.*, 2005). Vectors targeted to specific cell types are also being designed, including the use of tissue specific promoters (Cannon *et al.*, 1996; Certo *et al.*, 1998) and natural or engineered envelope proteins to target specific cell types. The site specific integration machinery of bacteriophage ϕC31 has been utilised in non-viral vectors to achieve targeted integration in murine and human cells (Groth *et al.*, 2000; Olivares *et al.*, 2002, Ortiz-Urda *et al.*, 2002). Incorporating the ϕC31 integrase system into viral vectors in order to avoid insertional mutagenesis is therefore a clear possibility.

1.3.2 Haematopoietic Stem Cells

Stem cells are characterised by their capacity for self-renewal and their multipotential nature. Examples of stem cells include haematopoietic, embryonic, neural, epithelial and mesenchymal stem cells. Stem cells were previously thought to be able to give rise only to cells that are in the tissues in which they reside, but are now thought to be able to generate cells of different tissues, referred to as "plasticity". However, in order to differentiate into different cell types, they must first home to the appropriate microenvironment (Tavassoli & Minguell, 1991; Watt & Hogan, 2000). Hence, the stem cells have the ability to leave their tissue niche and circulate in the blood stream, known to occur with HSCs (Siena *et al.*, 1989; Shields & Andrews, 1998) and MSCs (Fernandez *et al.*, 1997; Erices *et al.*, 2000; Reading *et al.*, 2000). However, plasticity of stem cells is still under much debate, mainly due to limited techniques to assess the *in vitro* or *in vivo* differentiation potential.

Haematopoietic stem cells are perhaps the best characterised stem cells (Lemischka *et al.*, 1986; Sachs, 1987; Spangrude *et al.*, 1988). They usually reside within the bone marrow, but can be mobilised using cytokines (G-SCF) into the peripheral blood, known as mobilised peripheral blood derived stem cells. Another source of HSCs is umbilical cord blood, which has the highest frequency of cells capable of reconstituting the immune system of a NOD-SCID mouse (Wang *et al.*, 1997). True HSCs can generate all the different cells in the blood (Figure 1.8), including immune cells such as B cells, T cells, NK cells, macrophages, dendritic cells, granulocytes and also erythrocytes (Hay, 1966). Their multipotential nature has been further evaluated, and some research groups believe that HSCs are also capable of

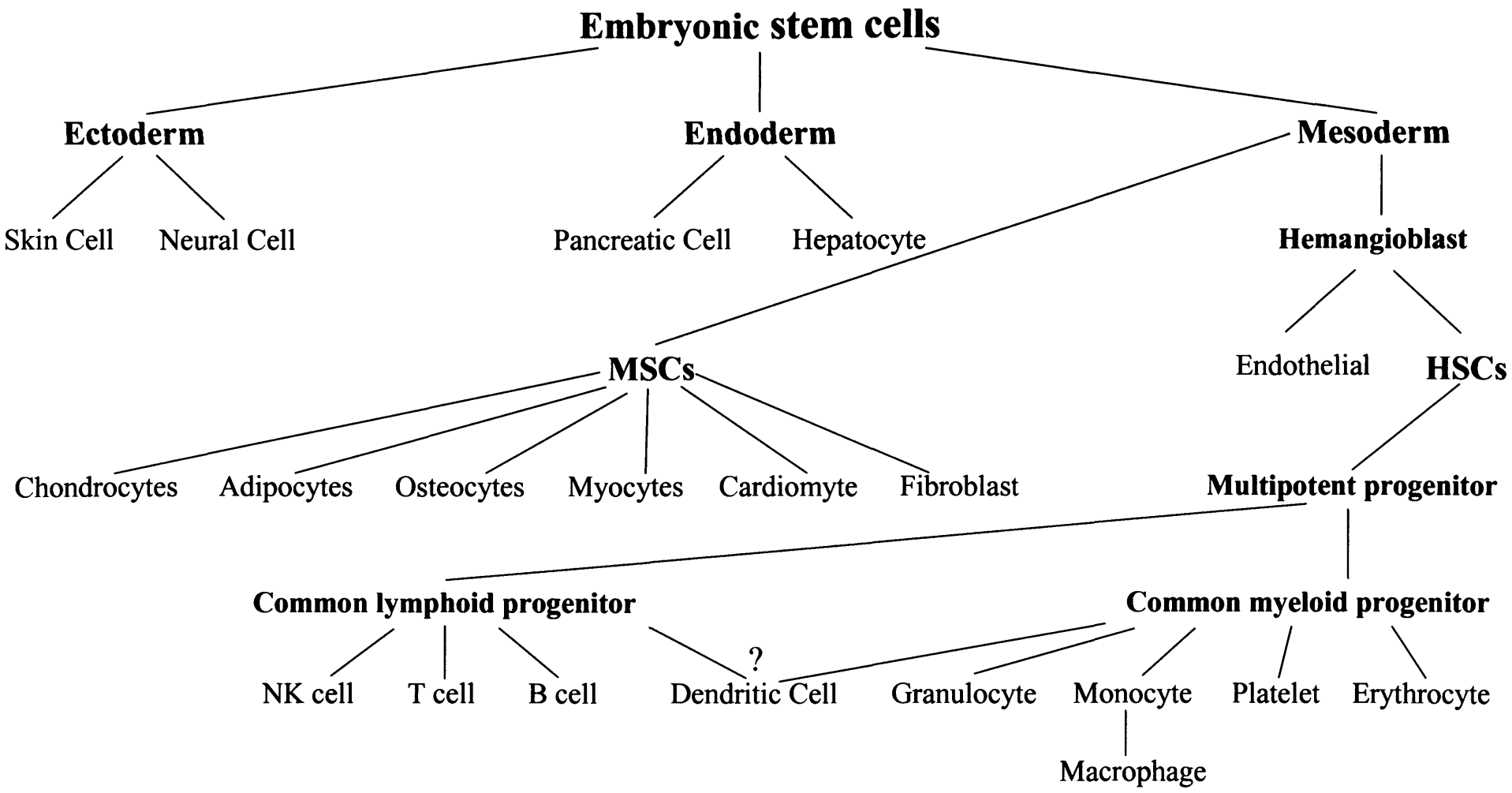


Figure1.8. Schematic drawing to show the origin of mesenchymal and haematopoietic stem cells and examples of the daughter cells they generate. Endoderm generates the epithelial cells of the digestive system, the mesoderm generates the supporting muscular and fibrous elements and the ectoderm gives rise to the epidermis and the entire nervous system.

differentiating into other cells such as hepatocytes (Lagasse *et al.*, 2000) and heart muscle cells (Orlic *et al.*, 2001). However, these reports of transdifferentiation are still a point of debate, and are contradicted by observations of other research teams who have failed to reproduce these data (Wagers *et al.*, 2002; Murry *et al.*, 2004; Balsam *et al.*, 2004). Nevertheless, the main interest in using HSCs in gene therapy is due to the well known ability of these cells to repopulate the haematopoietic system. Disadvantages of using HSCs include low rates of transduction as a result of their quiescent state and lack of cell surface receptors. This has resulted in a large amount of research into the use of cytokines to induce proliferation in order to facilitate gammaretroviral transduction. However, this activation also leads to differentiation into more committed haematopoietic progenitors which reduces the chances of targeting stem cells. Another drawback is that the most commonly used marker to isolate HSCs is CD34, and questions have been raised as to whether these cells are true stem cells as $CD34^-$ cells have also been found to possess properties of stem cells and CD34 is expressed reversibly on murine stem cells (Goodell *et al.*, 1996; Zanjani *et al.*, 1998; Bhatia *et al.*, 1998; Sato *et al.*, 1999) (in this report, the term haematopoietic stem cells will be utilised although the cells used in experiments in this study have not been proven to be true stem cells and may sometimes represent more committed progenitor cells). The most widely accepted assay to test for the presence of HSCs is to analyse their ability to reconstitute the haematopoietic system of a myeloablated host, usually the NOD-SCID mouse (hence the cells are also termed SCID repopulating cells). This is because haematopoietic reconstitution requires both extensive self-renewal of the transplanted HSC as well as differentiation into all mature blood types.

The most successful gene therapy trial to date using HSCs has been for X-linked SCID in a Paris study of 11 boys (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002). This trial involved the gammaretroviral transfer of the common gamma chain gene into autologous bone marrow derived HSCs. 9 of the 11 boys showed good immunological reconstitution, with transduced B, T and NK cells persistently detected in the peripheral blood. This resulted in significant clinical improvement with resolution of infectious complications and other chronic symptoms from the SCID (discussed in section 1.2.5.1). However three of these boys later developed

acute lymphocytic leukaemia as a direct result of the gene therapy procedure (discussed in more detail in section 8.2.1).

1.3.3 Mesenchymal Stem Cells

Mesenchymal stem cells are multipotential cells (Figure 1.8) capable of self-renewal. They are non-haematopoietic stem cells prevalent within the adult bone marrow where they form part of the connective tissue network, the bone marrow stroma. In this environment, MSCs secrete several haematopoietic and non-haematopoietic growth factors, and are therefore thought to be important for the formation and function of the stromal microenvironment. Mesenchymal stem cells were first discovered in 1976 by Friedenstein and colleagues, and are sometimes also referred to as fibroblast colony forming unit (CFU-F), marrow stromal cells or mesenchymal progenitor cells. MSCs were originally isolated by their adherence to tissue culture plastic, and have a spindle-shaped fibroblast like morphology. They have an average frequency of 1 cell per 10^4-10^5 bone marrow mononuclear cells (Friedenstein *et al.*, 1970; Gronthos & Simmons, 1996).

Some groups have published observations of a somewhat heterogeneous population of MSCs. Mets & Verdonk (1981) were the first to publish these observations and termed the different populations type I and type II cells (now called RS1 and RS2 respectively). RS1 cells are described as spindle shaped, rapidly dividing cells, and RS2 cells as being broader, dividing slowly if at all. During subcultivation, the appearance of RS2 cells formed an increasing fraction of the total cell population. (Bruder *et al.*, 1997). They noted that the spindle shaped cells have a higher replicative capacity, however the broad cells were not terminally differentiated cells, as they were still capable of differentiation. Since then, other groups have also demonstrated similar heterogeneity within the MSC population (Colter *et al.*, 2000; Sekiya *et al.*, 2002). It is also believed by some groups, that MSCs consist of a heterogeneous population of cells with different cells being uni-, bi- or multipotential cells. Owen and Friedenstein (1988) initially proposed this, and since then other research groups have also supported this theory (Kuznetsov *et al.*, 1997; Pittenger *et al.*, 1999). It is therefore clear that the properties of the MSCs have still not been fully elucidated, and different groups report contradicting observations regarding

their functional potential. Thus, it is still a point of controversy as to whether MSCs are truly stem cells, as their self-renewal capacity has still not been demonstrated (hence the different names for these cells as some groups are reluctant to call them mesenchymal stem cells). In this report, the cells will be termed mesenchymal stem cells although it has not been proven that they are indeed true stem cells.

Some of the differences reported could be due to different isolation and culturing methods. Purification of MSCs using STRO-1 and VCAM (Gronthos *et al.*, 2003) as well as D7-FIB positive selection followed by $CD45^{low}$ selection (Jones *et al.*, 2002) has been attempted in order to try to obtain more pure populations. This sort of purification may be preferential for gene therapy purposes as it would reduce the time required for MSC isolation. However these methods are generally not as widely accepted as the plastic adherence method, as they could give rise to different types of cells.

Mesenchymal stem cells have also been isolated from sources other than the bone marrow. Cells that appear to be similar to MSCs have been reported to be derived from muscle (Williams *et al.*, 1999), adipose (Loncar, 1992), bone (Nuttall *et al.*, 1998), and other organs. Particular interest has been paid to mesenchymal stem cells which have been derived from umbilical cord blood (Erices *et al.*, 2000; Romanov *et al.*, 2003; Lee, O.K. *et al.*, 2004; Gang *et al.*, 2004; Lee M.W. *et al.*, 2004). These MSCs are thought to perhaps have a greater differentiation potential than bone marrow derived MSCs. This was demonstrated by Lee, O.K. *et al.*, who differentiated MSCs into adipocytes, osteocytes, chondrocytes, hepatic cells and neuronal cells with a measurable action potential. Therefore, MSCs from an UCB source could be a promising therapeutic target. However, their presence and frequency in cord blood is still debated (Mareschi *et al.*, 2001; Wexler *et al.*, 2003; Yu *et al.*, 2004).

Flow cytometric analyses have failed to identify specific markers for MSCs as they have been found to exhibit phenotypic markers of mesenchymal, endothelial, epithelial and muscle cells. MSCs do not express the common leukocyte marker CD45, the haematopoietic marker CD34, or the markers CD14, CD33, CD117 or CD133. However, they do express the markers CD10, CD13, CD28, CD29, CD44,

CD73 (SH3 & SH4), CD90, CD105 (SH2 – an epitope of endoglin), CD166, LNGFR and STRO-1 (Haynesworth *et al.*, 1992; Pittenger *et al.*, 1999; Colter *et al.*, 2000; Guo *et al.*, 2001; Suva *et al.*, 2004). Therefore, to confirm the identity of these cells, their potentials to differentiate into other tissues is generally demonstrated rather than the presence of specific markers.

Following the pioneering work by Friedenstein *et al*, it was shown by several groups that MSCs can differentiate into cells of the mesenchymal lineage, such as adipocytes, osteocytes and chondrocytes, as shown in Figure 1.8. (Piersma *et al.*, 1983; Piersma *et al.*, 1985; Howlett *et al.*, 1986; Friedenstein *et al.*, 1987; Mardon *et al.*, 1987; Owen & Friedenstein, 1988; Keating *et al.*, 1990; Caplan, 1991; Haynesworth *et al.*, 1992; Beresford *et al.*, 1992; Cheng *et al.*, 1994.; Rickard *et al.*, 1994; Clark & Keating, 1995; Prockop, 1997; Pittenger *et al.*, 1999). Pittenger *et al.* importantly also showed that MSCs derived from individual stem cells retained the potential to differentiate along these lineages. MSCs have also been shown to differentiate into several other cell types including cardiomyocytes (Makino *et al.*, 1999; Toma *et al.*, 2002; Shake *et al.*, 2002; Mangi *et al.*, 2003) and also into non-mesenchymal neuronal cells *in vitro* (Sanchez-Ramos *et al.*, 2000; Kohyama *et al.*, 2001; Kim *et al.*, 2002; Jiang *et al.*, 2003) and *in vivo* (Azizi *et al.*, 1998; Kopen *et al.*, 1999).

In vivo MSC differentiation has been more difficult to demonstrate than the *in vitro* differentiation. However, Prockop *et al.*, 1997, showed that MSCs can repopulate various tissues in mice following X-ray irradiation. MSCs, containing a mutated gene for type I collagen, were detected in bone, cartilage, marrow and spleen at a frequency of between 1 and 12%. Hence, MSCs can make important contributions to mesenchymal tissues *in vivo*.

Since their discovery, MSCs have been extensively studied because of their powerful *ex vivo* expansion and multilineage differentiation potentials, and have already been used extensively in different therapies. The first clinical trial using MSCs showed that it was feasible and safe to systemically infuse *ex-vivo* expanded autologous MSCs as they were well tolerated (Lazarus *et al.*, 1995). Caplan and colleagues also demonstrated that MSCs can aid in the repair process when implanted into poorly

healing bone. They injected MSCs locally, which were found to promote the repair of surgical incisions in the knee cartilage of rabbits (Caplan, 1990; Goldberg & Caplan, 1994; Bruder *et al.*, 1994; Wakitani *et al.*, 1994).

Mesenchymal stem cells have also been utilised in clinical trials for Osteogenesis Imperfecta (OI). OI is an inherited disorder caused by a defective type I collagen in osteoblasts. This leads to osteopenia, multiple fractures, severe bone abnormalities and a shortened stature. Pereira *et al.* (1998) demonstrated in a murine model, that transplantation of normal mesenchymal progenitor cells resulted in a greatly improved phenotype of the OI mouse. Based on these results, a human study was subsequently initiated by Horwitz and co-workers (Horwitz *et al.*, 1999). Three patients were recruited for the study, where they transplanted unmanipulated marrow from sibling donors, following ablative conditioning of the patients. It was revealed that marrow derived osteoblasts precursors (presumed to be MSCs) migrated to the bones in all three children. Dense bone formation and a significant increase in total bone mineral content could be detected as early as three months following the transplant. An increase in growth rate and a reduction in the number of bone fractures were also observed. This study had important implications for future gene therapy trials using MSCs, as it was demonstrated for the first time in humans that MSCs were capable of engrafting, differentiating and normal functioning *in vivo*. Similarly, a clinical trial was carried out using purified MSCs (Horwitz *et al.*, 2002), which also revealed modest engraftment levels.

In addition, MSCs have recently been considered to treat metachromatic leukodystrophy and Hurler's Syndrome (Koc *et al.*, 2002), myocardial infarction (Caparrelli *et al.*, 2001; Shake *et al.*, 2002; Mangi *et al.*, 2003; Chen *et al.*, 2004) and cancer (Studeny *et al.*, 2002). Further potential uses of MSCs, which have only recently been discovered, are related to their apparent immunomodulatory functions. Mesenchymal stem cells have been described to induce a more tolerogenic phenotype on dendritic cells, naïve and effector T cells and natural killer cells, which have come into contact with MSCs (Aggarwal & Pittenger, 2004). They have also been shown to inhibit T cell proliferation by arresting the cells at the G1 phase of the cell cycle (Glennie *et al.*, 2005). Importantly, LeBlanc *et al.* (2003) also demonstrated *in vitro* that differentiated MSCs do not have an increased antigenicity.

MSCs have also been shown to suppress lymphocyte proliferation *in vitro* (Bartholomew *et al.*, 2002; Di Nicola *et al.*, 2002) and prolong skin graft survival *in vivo* (Bartholomew *et al.*, 2002), and have consequently been shown to suppress dendritic cell maturation and induce T-cell unresponsiveness (Zhang, W., *et al.*, 2004; Deng *et al.*, 2004; Beyth *et al.*, 2005, Aggarwal & Pittenger, 2005). Another interesting finding was that even xenogenic transplantation of MSCs was well tolerated, as demonstrated by Saito *et al.*, 2002. They showed that murine MSCs were capable of engrafting into injured myocardium of rats. This may suggest that universal donors of MSCs could be utilised in clinical trials. These findings have prompted more research into the use of MSCs in the treatment of graft versus host disease (GVHD) (Ringden *et al.*, 2002). Le Blanc *et al.* (2004) reported that a patient with severe treatment resistant grade IV acute GVHD of the gut and liver was transplanted with haploidentical MSCs and one year later appears to be clinically well. Hence, it has been suggested that MSCs may in the future be used not only to prevent graft versus host disease, but also to treat organ-transplant rejection and autoimmune disorders (Frank & Sayegh, 2004).

MSCs can also be used to introduce genes systemically, important for gene therapy of ADA-SCID. As ADA deficiency has been extensively studied, it has become apparent that it is a more systemic disease than other forms of SCID, most likely as a result of the ubiquitous nature of the ADA enzyme. It is therefore thought that the transduction and reinfusion of MSCs together with HSCs could offer a more systemic treatment than the use of HSCs alone as in current ADA gene therapy trials. Mesenchymal stem cells also have several other advantages over other stem cells currently used for gene therapy, such as haematopoietic and neural stem cells. MSCs can be easily expanded in culture without activation or differentiation, whereas both HSCs and neural progenitor cells have been found to be difficult to culture and expand *ex vivo*. MSCs also maintain their phenotype after expansion and maintain protein expression of the transgene for at least 6 months *in vitro* and for at least 3 months *in vivo* (Lee *et al.*, 2001). Moreover, large volumes of bone marrow are required to obtain adequate numbers of HSCs, and MSCs are clearly more easily accessible than neural stem cells (Glimm & Eaves, 1999; Carpenter *et al.*, 1999). Importantly, mesenchymal stem cells do not only differentiate into cells of the mesenchymal lineage, but also into neural cells which may help correction of

neurological abnormalities of ADA deficient patients (Hirschhorn *et al.*, 1980; Rogers *et al.*, 2001).

MSCs may also have the ability to serve as a feeder layer for the growth of HSCs as reported for both human and murine MSCs *in vitro* (Dexter & Testa, 1976; Gartner & Kaplan, 1980; Quesenberry & Lowry, 1992; Sutherland *et al.*, 1993; Deryugina & Muller-Sieburg, 1993; Haynesworth *et al.*, 1996; Gordon *et al.*, 1996; Majumdar *et al.*, 1998; Dormady *et al.*, 2001). Majumdar and colleagues (1998), for example, conducted a study analysing the ability of MSCs to maintain haematopoiesis in LTBMC (long term bone marrow culture) with $CD34^+$ haematopoietic progenitors. In particular, they examined the expression of cell surface molecules, cytokines and growth factors by MSCs. They found that the MSCs expressed a wide array of cytokines some of which act on haematopoietic cells, and from methylcellulose haematopoietic colony assays they were able to determine that MSCs were capable of maintaining and supporting HSC differentiation of purified $CD34^+$ cells. Murine studies have demonstrated that co-transplantation of human MSCs together with HSCs accelerates the haematopoietic recovery following bone marrow transplantation (Nolta *et al.*, 1994 & 2002: Brouard *et al.*, 1998; Noort *et al.*, 2002; Bensidhoum *et al.*, 2004). The haematopoietic support by the MSCs has been similarly demonstrated in humans (Koc *et al.*, 1999, 2000, 2001), and it is a feature which would make the use of MSCs ideal for ADA gene therapy in combination with HSCs. However, the exact identity of the cells capable of supporting the HSCs is still being debated (Minguell *et al.*, 2001). Therefore, if MSCs are to be used in the clinic, it is vital that the potentials of the cells in terms of engraftment and haematopoiesis support are well characterised, which may require a more precise study into the isolation procedure and culture conditions of these cells.

It is therefore clear that the use of mesenchymal stem cells in gene therapy for ADA deficiency may have several significant advantages, including their substantial expansion potential, differentiation potential and HSC support. However, it is crucial that the cells are extensively characterised, including their differentiation potential according to isolation procedure and cell culture conditions, to ensure that the most multipotential cells are utilised. This is particularly important for a multi-organ

disease such as ADA-SCID, which is currently being treated with gene therapy protocols that may not offer systemic treatment.

1.4 Statement of Aims

The aims of this study are to:

1. Assess immune function of patients treated with PEG-ADA.
2. Construct a gammaretroviral vector expressing ADA and evaluate its efficiency in transduction of $CD34^+$ cells for use in clinical gene therapy trial.
3. Assess immune recovery of ADA deficient gene therapy patient in comparison to ADA deficient patients treated with PEG-ADA.
4. Construct a lentiviral vector expressing ADA and evaluate its gene transfer efficiency in comparison to the gammaretroviral vector.
5. Generate mesenchymal stem cells and evaluate their differentiation potential.
6. Investigate the possibility of a systemic treatment for ADA deficiency using ADA-lentiviral transduced $CD34^+$ cells and MSCs.

2

MATERIALS AND METHODS

2.1. REAGENT SUPPLIERS

Chemicals were obtained from Sigma (Mo, USA), cell culture media from Invitrogen (Ca, USA), and restriction endonucleases and DNA modifying enzymes were obtained from Promega (Wi, USA) unless otherwise stated.

2.2 DNA MANIPULATIONS

2.2.1 Cloning

The human ADA gene was cloned by PCR amplification from the pOTB7 ADA vector (kind gift from Prof D. Valerio, Department of Gene Therapy, University of Leiden, Netherlands) using primers containing *XbaI* sites (forward primer: ATG TCT AGA ACC ATG GCC CAG ACG CCC, reverse primer: CGT CTA GAT CAG AGG TTC TGC CCT GCA G). These restriction sites were utilised to insert the gene into the gammaretroviral or lentiviral vector, using an intermediate pBluescript vector in order to facilitate the cloning procedure.

To create the pBlueScript-SFFV-ADA-WPRE, the SFFV-eGFP-WPRE fragment was replaced by SFFV-ADA-WPRE (The WPRE was a kind gift from Thomas Hope, University of Illinois, USA). The pBS-SEW plasmid was therefore digested with *BamHI* and *NotI*. These restriction sites were then blunted using Klenow large fragment DNA polymerase I (Invitrogen), and the vector religated using T4 DNA ligase. The pBS vector was cut using *XbaI* so that the hADA cDNA with *XbaI* linkers could then be inserted (refer to Figure 4.1).

For construction of the gammaretroviral-ADA vector, the gp91phox gene was removed from the gammaretroviral vector by digestion using *EcoRI* and *NotI*, and the vector was then blunted. The pBS-SAW vector was digested using *XbaI* and *XhoI* to release the ADA-WPRE fragment, which was then ligated into the gammaretroviral vector, already containing SFFV LTR to create the SFada/W vector (refer to Figure 4.1C).

The lentiviral-ADA vector was constructed by digesting pHR.Sin.cPPT.SEW with *EcoRI* and *KpnI* to remove the SEW fragment. The pBS-SAW was also digested

using *EcoRI* and *KpnI* to release the SAW fragment, which was then directly ligated into the lentiviral backbone (refer to Figure 6.1A).

2.2.2 Preparation of DH5α Cells

500mL Lennox L-Broth (LB) was inoculated using 5 mL of an overnight culture of the electro-competent *E. coli* strain DH5α (Promega). The cells were incubated at 37°C in a shaking incubator for approximately four hours until the absorbance at 600nm was approximately 0.8. To harvest, the flask was chilled on ice for 30 minutes and the cells were centrifuged at 4,000xg for 15 minutes at 4°C. The bacterial pellet was resuspended in 500mL sterile 10% glycerol and centrifuged as previously. The pellet was then serially resuspended in 250mL, 10mL and approximately 1mL of 10% glycerol so that the final cell concentration was approximately $3x10^{10}$ cells/mL. The bacterial suspension was aliquoted into 50μL samples and stored at -80°C.

2.2.3 Plasmid Preparations

DH5α cells were used to transform the gammaretroviral plasmid and the three lentiviral plasmids, all containing ampicillin resistance genes. Salts from the ligation buffer were removed from the plasmid preparation by dialysis on 0.25μM nitrocellulose 10mm disc filters (Millipore, MA, USA) on distilled water for 20 minutes. The plasmid solution was then added to 50μL of DH5α cells on ice in a 2mm gap cuvette (Biorad, CA, USA), and was electroporated using the Gene Pulser Electroporator (Biorad) at 2.5kV, 200Ω, 25μF. Approximately 950μL LB medium was added to the cells, which were incubated for 30-45 minutes at 37°C in a shaking incubator. The cells were plated on LB agar plates containing 100μg/mL ampicillin and incubated overnight at 37°C, after which single colonies were used to inoculate 5mL LB with 100μg/mL ampicillin.

The plasmids were purified from the 5mL cultures using a Miniprep kit (Qiagen, KJ Venlo, The Netherlands) according to the manufacturers' instructions, to screen for transformed colonies by restriction enzyme digests. Briefly, the bacterial cells were pelleted and the plasmid DNA was extracted by alkaline lysis in the presence of

RNaseA. The lysates were neutralised and adjusted to high salt conditions prior to loading onto the silica-gel column. The column was washed and then eluted in low salt buffers. Once the plasmids had been analysed by restriction digests, 500μL of the transformed cultures was used to inoculate 500mL of LB-ampicillin for large scale plasmid preparations (Maxi Kit for the gammaretrovirus and Mega Kit for the lentiviral plasmids, both from Qiagen). Following overnight incubation at 37°C in a shaking incubator, the cells were pelleted by centrifugation at 4°C and resuspended in the presence of RNaseA. The plasmid DNA was purified by alkaline lysis of the bacteria, and the lysate was neutralised and filtered. The DNA was then bound to the anion-exchange resin column under low salt and pH conditions. The column was washed to remove unbound fractions and the plasmid was eluted using a high salt solution (1.6M NaCl) in 15% isopropanol, at pH7. The DNA was desalted by precipitation with isopropanol, and washed in 70% ethanol. The plasmid was resuspended in Tris-EDTA (TE) buffer and the DNA concentration was estimated by absorbance at 280nm using a GeneQuant machine (Pharmacia/Pfizer, NY, USA). The ratio of 280:260nm was then used to determine the purity of the DNA, with the absorbance ratio of 1.8 indicating a pure sample with minimal protein contamination.

2.2.4 Sequencing of Plasmids

As the ADA gene was amplified by PCR, the sequence was verified by sequencing using DYEnamic ET dye terminator kit (Amersham, Little Chalfont, UK). The DNA to be sequenced was diluted to 1μg /10μL. The DNA was mixed with the primer (at 5μM) and the DYEnamic ET terminator reagent premix. The samples were placed in a thermal cycler, and the following 25 cycle programme was initiated: 20 seconds at 95°C, 15 seconds at 50°C and 1 minute at 60°C. Unincorporated dye-labelled terminators were removed by isopropanol precipitation, and the reaction products were resuspended in a formamide loading solution for separation and detection on the MegaBASE 1000 sequencing machine.

The gammaretroviral vector that was used for the clinical gene therapy trial (SFada/W) was sequenced by Lark Technologies from the 5' LTR to the 3' LTR inclusive.

2.3 CELLS AND CELL CULTURE

All cells were incubated in humidified chambers at 37°C with 5% CO_2. The cells utilised are listed in Table 2.1, and the media in which they were cultured in Table 2.2. Table 2.3 displays the antibodies used for flow cytometry analysis and Western blotting. Flow cytometry was performed utilising a Becton Coulter Epics XL, with the aid of the Expo 32 (Becton Dickinson, NJ, USA) acquisition and analysis software.

Cells	Origin	Supplier	Medium
Phoenix	293T Human Embryonic Kidney	Nolan Laboratories, Stanford, USA	DMEM
PG13	NIH 3T3 Mouse Fibroblast	M. Collins, UCL, London	DMEM
HeLa	Human Cervical Carcinoma	ECAAC, Salisbury, UK	DMEM
293T	Human Embryonic Kidney	ATCC, USA	DMEM
Jurkats	Human Leukaemic T cell	Molecular Haematology, ICH, London	RPMI
Fibroblasts – Patient	Primary Skin Fibroblasts	H. Gaspar, ICH, London	DMEM
Fibroblasts – Normal	Primary Skin Fibroblasts	M. Hubank, ICH, London	DMEM
Patient LCLs	Peripheral Blood		RPMI
$CD34^+$s	Mobilised Peripheral Blood or bone marrow		X-Vivo 10
MSCs	Bone Marrow		DMEM

Table 2.1. Cells and media used in this study.

Abbreviations: ECAAC; European Collection of Cell Cultures; LCL, Lymphoblastoid cell line; MSCs, mesenchymal stem cells.

Media Name	Medium	Supplement	Media Supplier
Complete DMEM	DMEM with Glutamax	10% FCS, 10units/mL Pen– 10μg/mL Strep	Invitrogen
Complete RPMI	RPMI with Glutamax	10% FCS, 10units/mL Pen– 10μg/mL Strep	Invitrogen
CD34 Gammaretroviral Activation Media	X-Vivo 10	1% HSA, 300ng/mL hSCF, 20ng/mL hIL-3, 300ng/mL hFlt-3, 100ng/mL hTPO	Bio Whittaker, NJ, USA
CD34 Gammaretroviral Transduction Media	X-Vivo 10	1% HSA, 600ng/mL hSCF, 40ng/mL hIL-3, 600ng/mL hFlt-3, 200ng/mL hTPO	Bio Whittaker
CD34 Lentiviral Transduction Media	X-Vivo 10	1% HSA, 100ng/mL hSCF, 100ng/mL hFlt-3, 20ng/mL hIL-6, 20ng/mL hIL-3	Bio Whittaker

Table 2.2. Compositions of media used in this study.

Abbreviations: DMEM, Dulbeccos' Modified Eagle Medium (Invitrogen); FCS, Foetal calf serum (Sigma); Pen-Strep, Penicillin-Streptomycin (Invitrogen); RPMI, Roswell Park Memorial Institute (Invitrogen); HSA, human serum albumin (Bio Products Laboratory); hSCF, human stem cell factor; hIL-3, human interleukin 3; hFlt-3 *fms*-like tyrosine kinase 3; hTPO, human thrombopoietin (all from R & D Systems).

Name/ Specificity	Conjugate	Application	Species raised in	Concentration/ Dilution	Source
αADA	–	FC	Mouse	5μg/100μL	Hershfield
αMouse IgG1	Biotin	FC	Rat	0.5μg/100μL	Pharmingen
Streptavidin	PE	FC	Mouse	1:100	Pharmingen
αADA	–	Western Blot	Goat	1:100	Santa Cruz, CA, USA
αgoat	HRP	Western Blot	Donkey	1:1,000	Serotec, Oxford, UK
Isotype IgG1	FITC	IP	Mouse	1:20	BD
Isotype IgG1	PE	IP	Mouse	1:20	BD
Isotype IgG1	PerCP	IP	Mouse	1:20	BD
αCD3	FITC	IP	Mouse	1:20	BD
αCD3+16+56	FITC+PE	IP	Mouse	1:20	BD
αCD4	PE	IP	Mouse	1:20	BD
αCD4	PerCP	IP	Mouse	1:20	BD
αCD8	PE	IP	Mouse	1:20	BD
αCD8	CY	IP	Mouse	1:20	BD
αCD19	PE	IP	Mouse	1:20	BD
αCD27	FITC	IP	Mouse	1:20	BD
αCD27	PE	IP	Mouse	1:20	BD
αCD34	FITC	IP	Mouse	1:20	BD
αCD45	PerCP	IP	Mouse	1:20	BD
αCD45RO	FITC	IP	Mouse	1:20	BD
Isotype IgG1	FITC	MSC FC	Mouse	1:20	BD
Isotype IgG1	PE	MSC FC	Mouse	1:20	BD
Isotype IgG1	PerCP	MSC FC	Mouse	1:20	BD
Isotype IgG2a	PE	MSC FC	Mouse	1:20	eBioscience, CA, USA
Isotype IgG2b	FITC	MSC FC	Rat	1:20	eBioscience
αCD13	PE	MSC FC	Mouse	1:20	BD
αCD14	FITC	MSC FC	Mouse	1:20	BD
αCD29	PE	MSC FC	Mouse	1:20	BD
αCD33	PE	MSC FC	Mouse	1:20	BD

αCD34	FITC	MSC FC	Mouse	1:20	BD
αCD44	FITC	MSC FC	Rat	1:100	eBioscience
Name/ Specificity	Conjugate	Application	Species raised in	Concentration/ Dilution	Source
αCD45	PerCP	MSC FC	Mouse	1:20	BD
αCD73 (SH4)	PE	MSC FC	Mouse	1:20	BD
α CD90	FITC	MSC FC	Mouse	1:20	Serotec
αCD105	FITC	MSC FC	Mouse	1:20	Serotec
αCD106	PE	MSC FC	Mouse	1:20	BD
SH2	-	MSC FC	Mouse	1:20	ATCC, VA, USA
αMouse	FITC	MSC FC	Rat	1:100	eBioscience
αGFAP	-	MSC Conf	Mouse	1:200	BD
αNSE	-	MSC Conf	Mouse	1:100	Santa Cruz
αTau-2	-	MSC Conf	Mouse	1:200	BD
αNeuN	-	MSC Conf	Mouse	1:100	Chemicon, CA, USA
αMouse	FITC	MSC Conf	Rat	1:100	eBioscience
Phalloidin	Alexa 488	MSC Conf	-	1:20	Molecular Probes, Invitrogen
αCollagenII	-	MSC IHC	Mouse	1:100	NeoMarkers/ Labvision, CA, USA

Table 2.3. Antibodies used in this study.

Abbreviations: FC, flow cytometry; PE, Phycoerythrin; HRP, Horseradish Peroxidase; FITC, Fluorescein isothiocyanate; IP, immunophenotyping; BD, Becton Dickinson; PerCP, Peridinin chlorophyll protein; Conf, Confocal; IHC, immunohistochemistry.

2.3.1 Generation of Epstein Barr Virus Immortalised Lympho-blastoid B Cell Line

Lymphoblastoid cell lines were generated from patient blood following informed consent. The patient blood sample (in EDTA) was mixed with an equal volume of RPMI 1640. The peripheral blood mononuclear cells (PBMCs) were isolated by

density gradient (1.077g/mL Ficoll-Paque from Pharmacia), by centrifugation at 1,000xg for 20 minutes. The cells were washed twice in RPMI/20% FCS before resuspending approximately $3x10^6$ cells in 100 μL RPMI/20% FCS, mixed with concentrated Epstein Barr Virus (EBV) strain B95-8 (kind gift from Reza Sharifi). The cells were incubated with the virus at 37°C for 1 hour after which the volume was made up to 1mL by adding complete RPMI and the cells were transferred to a 24-well plate.

The LCLs were then maintained and expanded in complete RPMI. Once the LCL had been generated, three colour flow cytometry analysis was performed using the following lymphoid and leukocyte markers: CD3, CD19, CD45 and CD27 (all from BD Biosciences) to confirm the homogeneity of B cells. The relevant antibodies were incubated with the cells for 30 minutes at 4°C, followed by washing in PBS and fixing in 1% paraformaldehyde (PFA) in PBS.

2.3.2 Production of Mesenchymal Stem Cells

Human mesenchymal stem cells were isolated (following informed consent) from patient or normal donor bone marrow samples using a Ficoll-Paque density gradient centrifugation followed by plastic adherence to the tissue culture flask. Following two passages by trypsinisation the cells appeared homogeneous. Morphology of the cells was analysed using the Olympus IX70 inverted fluorescence microscope. The mesenchymal stem cells had a fibroblast-like morphology, and were characterised by their antigenic phenotype and their ability to differentiate into different mesenchymal lineages *in vitro* by controlled induction conditions.

2.3.3 MSC Antigenic Phenotyping

The mesenchymal stem cells were trypsinised and resuspended in 100 μL PBS. The appropriate antibodies (CD13, CD14, CD29, CD33, CD34, CD44, CD45, CD73 (SH4), CD90, CD105 (SH2), and CD106) at appropriate dilutions (see Table 2.3) were added and incubated for 20 minutes at room temperature. Following the staining, the cells were washed twice in PBS prior to being fixed in PBS/1% PFA and analysed in the flow cytometer.

Stain/Antibody/Assay	Specificity	Cell Type Identity
Oil Red O	Lipid Vacuoles	Adipocytes
Alizarin Red S	Mineralisation of ECM	Osteocytes
Calcium Deposition Assay	Calcium Content	Osteocytes
Toluidine Blue	Proteoglycans within ECM	Chondrocytes
Safranin O	Sulfated proteoglycans	Chondrocytes
Collagen II	Cartilage	Chondrocytes
GFAP	Astrocyte marker	Neural
Tau-2	Microtubule associated protein	Neural
NeuN	Neuronal nuclei	Neural
NSE	Glycolytic enzyme specific to nervous tissue	Neural

Table 2.4. Stains and antibodies used to analyse MSC differentiation.
Abbreviations: ECM, extracellular matrix

2.3.4 Adipogenic Differentiation of Mesenchymal Stem Cells

Mesenchymal stem cells were seeded in a 12-well plate at a confluency of 3000 cells/cm^2. Adipogenic differentiation was induced by the addition of 0.5mmoles/L 1-methyl-3-isobutylxanthine (Sigma), 1μmol/L dexamethasone, 5μg/mL insulin (Sigma), 60μmol/L indomethacine (Sigma), and 10% FCS to the media. The induction media was added to the cells every 2-3 days until the cells were confluent, usually after 2-3 weeks (Pittenger *et al.*, 1999). Mesenchymal stem cells grown in the absence of induction media constituted the non-induced control cells.

Following differentiation, the cells were analysed by observation of morphology and by immunohistochemistry. The adipocytes were stained with Oil Red O (Sigma) which is specific for lipid deposits (Table 2.4). The media was removed and the cells were rinsed with PBS prior to fixing by incubation with 10% formalin (Sigma) for 60 minutes at room temperature. The cells were then rinsed with sterile water followed by 60% isopropanol. The cells were incubated with 0.12% Oil Red O for 5 minutes at room temperature, after which they were washed with water. Hematoxylin

counterstain (Sigma) was added for 1 minute and the cells were then washed with water prior to being viewed using the Olympus IX70 inverted fluorescence microscope.

2.3.5 Osteogenic Differentiation of Mesenchymal Stem Cells

To induce osteogenic differentiation, mesenchymal stem cells were seeded as for adipogenic differentiation. For osteogenic differentiation, the MSCs were grown in media containing 0.1μmoles/L dexamethasone (Sigma), 10mmoles/L β-glycerol phosphate (Sigma), 0.05mmoles/L ascorbic acid (Sigma) and 10% FCS. The induction media was similarly added to the cells every 2-3 days until the cells were confluent (Pittenger *et al.*, 1999). Mesenchymal stem cells grown in the absence of induction media constituted the non-induced control cells.

Once the cells were differentiated, 2-3 weeks after the initiation of induction, the cells were analysed by observation of morphology and by immunohistochemistry. The osteocytes were also tested for calcium content by a dye-binding method. The calcium was extracted by incubating the cells in 0.5N HCl for 4 hours at 4°C with shaking. The cells were then centrifuged at 500xg for 2 minutes and the calcium containing supernatant was collected. Calcium was then detected using the Sigma Calcium Detection Kit and the colour change was detected by measuring the absorbance at 575nm using a spectrophotometer. The calcium concentration was calculated by extrapolation from a calcium standard curve.

The differentiated cells were stained for deposits of calcium salts using Alizarin Red S stain as described by Bodine *et al.*, 1996 (Table 2.4). For this, the osteocytes or non-differentiated control cells were washed in PBS and fixed in 10% formalin for ten minutes. Three washes with PBS were performed prior to staining with 2% Alizarin Red S for 2-5 minutes. The cells were then washed in PBS and visualised using the Olympus IX70 inverted fluorescence microscope.

2.3.6 Chondrogenic Differentiation of Mesenchymal Stem Cells

Mesenchymal stem cells were seeded at one million cells per well in a 96 well plate. Chondrogenic differentiation was induced by addition of 10ng/mL TGFβ3

(Peprotech) and 1% bovine serum albumin (Sigma) to high glucose media. Mesenchymal stem cells grown in the absence of induction media constituted the non-induced control cells. However, as they were found to be non-viable, tumour sections (gift from S. Barker) were used as control in the staining procedures

The chondrogenic cell pellet was fixed in 10% formalin for one hour at room temperature. The pellet was carefully transferred to a 15 mL falcon tube, and was dehydrated in successive ethanol washes of 70%, 70%, 80%, 80%, 95%, and 95% for 15 minutes in each concentration. The pellet was stained briefly in 1% eosin (in water) to aid visualisation of the small pellet which turns orange. Following the eosin stain, the pellet was washed 3x in 10% ethanol. The pellet was then transferred to a 5 mL bijou tube and incubated twice in histoclear for 20 minutes each time. The pellet was transferred to three successive incubations in 58°C paraffin for 20 minutes each. The pellet was embedded in a paraffin block, and ribbons of 5μm sections were cut. The sections were incubated in water at 40°C to straighten them, and they were then transferred to positively charged glass microscope slides. The slides with the sections were incubated at 60°C for one hour prior to being cooled for 10 minutes. The slides were stored at 4°C for future staining.

Chondrocyte sections were analysed by safranin-O staining, which reveals the proteoglycans, toluidine blue which stains proteoglycans within ECM and by immunostaining for type II collagen (Table 2.4). The sections were deparaffinized by three successive 3 minute washes in histoclear, followed by two successive 3 minute washes in 100% ethanol, two successive 3 minute washes in 95% ethanol and one wash in 70% ethanol. For the Safranin-O stain, the sections were incubated in Weigert's ion hematoxylin for 4 minutes, followed by destaining in fresh acid alcohol (1% HCl in 70% ethanol) and rinsing in water. The sections were stained with methyl green for 5 minutes and then with 0.1% aqueous Safranin O for 5 minutes. Following staining, the sections were dehydrated by two successive 5 minute washes in 95% ethanol, three successive 5 minute washes in 100% ethanol and three successive 5 minute washes in histoclear. Sections were mounted in DPX synthetic resin mounting medium for microscopy. For the Toluidine Blue staining, the sections were incubated in Toluidine Blue stain (1% Toluidine Blue in a 1%

NaCl solution, pH2), followed by 3 washes in water. The sections were then dehydrated quickly through 95% ethanol and 2 incubations in 100% ethanol, followed by 3 washes in histoclear. The sections were then mounted in DPX synthetic resin mounting medium for microscopy. For the immunostaining for type II collagen, the sections were deparaffinized as previously described and incubated in water for 5 minutes. Endogenous peroxidase activity was quenched by incubating the pellet sections with DAKO peroxidase block for 5 minutes, followed by washed in deionised water and wash buffer (Tris buffered saline, 0.05% Tween). The sections were then treated with proteinase K for 5 minutes followed by washes in deionised water and wash buffer. The sections were incubated with the primary antibody to type II collagen for 30 minutes after pepsin (1mg/ml) digest and then washed in wash buffer. The sections were incubated with peroxidase labelled secondary reagents and substrate chromogen (peroxidase-DAB) as per kit instructions (DAKO EnVision+ System HRP (DAB) kit) and counterstained with methyl green. The sections were then dehydrated in two successive 5 minute washes in 95% ethanol, three successive 5 minute washes in 100% ethanol and three successive 5 minute washes in histoclear. The sections were then mounted in DPX synthetic resin mounting medium for microscope viewing using the Olympus IX70 inverted fluorescence microscope.

2.3.7 Neuronal Differentiation of Mesenchymal Stem Cells

Mesenchymal stem cells were seeded in a 12-well plate at a confluency of 3000 cells/cm^2. Neuronal differentiation was induced by the addition of 20ng/mL hEGF and 20ng/mL hbFGF and 10% FCS to the media (Carpenter *et al.*, 1999). The induction media was added to the cells every 2-3 days until the cells were confluent, usually after 2-3 weeks. Mesenchymal stem cells grown in the absence of induction media constituted the non-induced control cells.

The cells were analysed for neuronal differentiation by fluorescent immuno-histochemistry staining for the specific neuronal markers GFAP, Tau-2, NeuN and NSE (Table 2.4). The cells were washed in PBS and fixed using 10% formalin for 10 minutes at 4°C. The cells were permeabilised in 0.5% v/v Triton X-100 for 5 minutes and washed three times in PBS. The cells were incubated for 20 minutes in 1% BSA prior to primary antibody staining (see Table 2.3) in block solution (1% BSA) for one hour. Following the staining, the cells were washed three times in PBS and

incubated with the secondary antibody (see Table 2.3) for 45 minutes in block solution. Excess antibody was removed by three washes in PBS followed by a wash in water. The slides were mounted using DAKO fluorescence mounting medium and the coverslips were sealed with nail varnish. A laser-scanning confocal microscope (Leica, St Gallen, Switzerland) fitted with the appropriate filters was used to image slides. Images were processed using Leica TCS start and Adobe Photoshop software, with the help of Mike Blundell.

2.3.8 Mesenchymal Stem Cell Homing in a Murine Model

NOD/ LtSz -scid/scid (NOD-SCID) mice (original stocks kindly provided by John E Dick, Hospital for Sick Children, Toronto) were housed in sterile microisolator cages in a laminar flow caging system (Thoren, Hazleton, PA) and supplied with sterile food, water and bedding. All manipulations were conducted in a laminar flow hood.

Mesenchymal stem cells were marked with eGFP-lentivirus as described in section 2.5.4. A proportion of cells were not transduced and served as negative controls. The transduced cells were analysed by flow cytometry to ensure that the cells had been successfully transduced. When this had been confirmed, the cells were trypsinised, washed in PBS and resuspended at a concentration of $0.5x10^6$ cells/200μL. Transduced or untransduced MSCs ($0.5x10^6$ cells) were injected intravenously via the tail vein in 7 to11 week old NOD-SCID mice that had been sublethally irradiated with 300cGy (^{137}Cs source). Six weeks following the injection of the MSCs, the mice were sacrificed and analysed for the presence of human MSCs in different organs. The organs analysed were blood, bone marrow, spleen, thyroid gland, liver, kidney, brain, heart, lung, and muscle. They were analysed for eGFP expression by flow cytometry and for the human β-actin gene by real time PCR.

For the flow cytometric analysis, single cell suspensions were generated from the organs. Blood and bone marrow were processed by lysing erythrocytes using lysing buffer (Sigma, containing 8.3g/L ammonium chloride) for 10-15 minutes at room temperature until the sample was clear. The sample was then washed in PBS and fixed in 1% PFA/PBS-0.5% BSA. Soft organs such as spleen, thymus, kidney and liver were homogenised and passed through a 70μM cell strainer to generate single

cell suspensions. Organs which were more fibrous, such as brain, heart, lung and muscle were incubated with 1g/L dispase (Invitrogen) prior to passing through a cell strainer. Briefly, the organ was cut into small pieces and digested by incubation in media containing dispase (RPMI 1640, 10% FCS, 1% dispase - Gibco). The organ pieces were incubated at 37°C for 30 minutes in a shaking incubator. The cells were homogenised and passed through a cell strainer, and any remaining pieces were further incubated in dispase medium. Once a single cell suspension was generated, with or without the aid of dispase, the cells were centrifuged at 300xg for 5 minutes. The supernatant was removed, and red blood cells were lysed by the addition of ammonium chloride for 7-10 minutes at room temperature. The samples were washed twice with PBS and then resuspended in an appropriate volume using 1% PFA/PBS-0.5% BSA and analysed by flow cytometry for eGFP.

For real time PCR analysis, a section of tissue less than 25mg was utilised for DNA extraction by the DNeasy kit (Qiagen). Genomic DNA was extracted from the cells as per manufacturer's instructions. Briefly, the tissue was digested by incubation with proteinase K. The lysates were loaded onto DNeasy spin columns containing silica gel membranes. The DNA binds selectively, and contaminants pass through. Residual contaminants were removed by two further washes, and the DNA was eluted in a low salt buffer. The samples were then analysed for the presence of the human β-actin gene using real time PCR analysis as described in section 2.6.7.

2.4 VIRUS PRODUCTION

2.4.1 Gammaretrovirus Production

Gammaretroviral particles were generated by transiently transfecting phoenix cells with the vector DNA in the presence of lipofectamine (Invitrogen) to aid uptake of DNA. Ecotropic Phoenix cells were plated at a density of $3x10^5$ cells per well in a 12-well plate. The following day the cells were transfected using the SFada/W plasmid. The diluted vector DNA at 1μg was combined with 10μg diluted lipofectamine 2000 (Invitrogen) and incubated for 20 minutes at room temperature. The DNA-lipofectamine was added to the cells in Optimem and incubated for 4

hours at 37°C after which the media was replaced with complete DMEM. Following 48 hours of incubation, the media was replaced.

The virus produced from the Phoenix cells was then used to stably transduce PG13 cells. The PG13 cells were plated at a density of $1x10^5$ cells per well in a 12 well plate the day before transduction. The supernatant from the Phoenix cells was collected and filtered through a 0.45μm filter. 1mL supernatant was added to 1mL complete DMEM and 8μg/mL polybrene (Sigma). The cells were transduced twice at 24-hour time intervals.

To generate gammaretrovirus for transductions of a range of cell types, the polyclonal PG13-ADA cells were seeded the day before harvesting. $5x10^6$ PG13-ADA cells were plated in a T75, and the cells were incubated at 37°C for approximately 8 hours. Once adhered, the media was removed from the cells and replaced with the minimal volume 7mL complete DMEM, and the cells were incubated overnight at 33°C, which is thought to improve gammaretrovirus stability. The gammaretroviral supernatant was collected and filtered through a 0.45μm filter prior to use in transductions.

2.4.2 Single Cell Fluorescence Activated Cell Sorting

$3x10^6$ cells of the polyclonal PG13 cell population transduced with SFada/W were sorted from a viable population using a Becton Coulter ALTRA fluorescent activated cell sorter (FACS) and the Expo 2 software. 150μL of a 1:1 ratio of filtered conditioned media: fresh media was added to each well in the 96 well plate into which each cell was sorted. Following the sort, the plates were spun at 500xg for 1 minute to pellet the cells. The sorted cells were then incubated at 37°C, and the viable cells were expanded in culture. Expression and activity of ADA were then evaluated for each single cell clone.

2.4.3 Lentivirus Production

Transfection of the lentiviral DNA was carried out on 293T cells, which were seeded in a 150 cm flask one day prior to transfection to be approximately 80% confluent on the day of transfection. The DNA was complexed to 1mM polyethylenimine (PEI)

(Sigma) by incubating 40μg of the transgene construct, 10μg of pMDG2 (vesicular stomatitis virus envelope glycoprotein (VSV-G)) expression vector and 30μg of p8.91 (HIV-1 gag-pol expression vector) with PEI for 20 minutes, before addition to the cells. The viral supernatant was harvested 48 and 72 hours after the transfection, filtered through a 0.45μm filter and then concentrated by centrifuging at 91,000xg for 2.5 hours. The virus was then resuspended in approximately 300μL X-Vivo 10.

2.4.4 Viral Titration Assay

The gammaretro- and lentivirus were titred on HeLa cells, which were seeded at a density of $1x10^5$ cells per well in a 6-well plate one day prior to transduction. The gammaretrovirus was used in 5 different concentrations, neat, 1:5, 1:25, 1:125, 1:625. The lentivirus was diluted 1:100, 1:1000, 1:10,000 and 1:100,000. The virus was added to the HeLa cells in the presence of 8μg/mL polybrene. The cells were incubated for 48 hours prior to analysis of transgene expression by flow cytometry. The titre of the virus was determined as below:

$$\frac{(\%\text{ positive cells}) \times (\text{number of cells infected})}{\text{Volume Supernatant (mL)} \times 100} = \text{Infectious virus particles/mL}$$

2.5 TRANSDUCTIONS

2.5.1 Gammaretroviral Transduction of ADA$^-$ Skin Fibroblasts

Viral supernatant was added to the fibroblasts at a 1:1 dilution with fresh media, supplemented with 8μg/mL polybrene, to enhance cell-virus contact and aid viral uptake. Three rounds of transductions were performed at 24-hour intervals. The fibroblasts were analysed for transgene expression by Western blotting (see section 2.6.2) and flow cytometry (see section 2.6.1) and for ADA activity (see section 2.6.3).

2.5.2 Gammaretroviral Transduction of ADA$^-$ B-Lymphoblastoid Cell Line

As for the skin fibroblasts, the patient LCLs were transduced with ADA gammaretrovirus to attempt reconstitution of ADA expression and activity *in vitro*. For virus production, the polyclonal PG13-ADA were grown in complete RPMI. The wells of a 24-well plate were coated with 10μg/mL fibronectin (Sigma) for 2 hours at 37°C, and were then washed with complete RPMI-1640. The fibronectin co-localises the cells and the virus and thus aids viral uptake. The wells were preloaded with virus, and the plate was centrifuged at 200xg for 10 minutes. 50,000-100,000 cells were added, followed by neat viral supernatant and 8μg/mL polybrene, after which the samples were centrifuged at 200xg for 10 minutes to pellet the cells. The cells were transduced a further two times with neat viral supernatant at 12-hour intervals, after which half the media was replaced with fresh complete RPMI.

2.5.3 Gammaretroviral Transduction of Haematopoietic Stem cells

Mobilised peripheral blood derived CD34$^+$ cells, obtained from the Department of Haematology, UCL, were transduced with a Good Manufacturing Practice (GMP) grade ADA-gammaretrovirus (produced by EUFETS, Germany) during a 5 day procedure. On day 1, the cells were activated using a cytokine mix to induce cell division upon which gammaretroviruses rely for nuclear entry. The cells were counted and resuspended in activation media (Refer to Table 2.2 for media composition) to give a cell density of $0.5x10^6$ cells/mL. Retronectin coated plates or X-fold cell-culture containers (Nexell, Irvine, CA, USA) were prepared on day two by adding 8μg/mL Retronectin (gift from Takara Bio Inc.) in PBS to the plates, which were left to incubate at room temperature for 2 hours. This was followed by a wash in PBS-1% HSA, after which the plates were maintained over-night in PBS at 4°C. On days 3, 4 and 5 the CD34$^+$ cells were transduced with the ADA gammaretrovirus and eGFP gammaretrovirus as a control. On day 3, the plate was washed with PBS-1%HSA, after which the Retronectin coated plate was pre-loaded with gammaretrovirus (enough to cover the surface of the plate or bag) for 30 minutes on a rocker. The cells were pelleted by centrifugation and then counted, after which they were resuspended in half volume transduction media (containing 2x the amount of cytokines and HSA present in the activation media), half volume of viral

supernatant to achieve a cell density of approximately $0.5x10^6$ cells/mL. The cells were then incubated at 37°C for approximately 20 hours. On day 5, the cells were transduced for five hours, after which the cells were analysed immediately, or the virus was removed and the cells resuspended in fresh media to maintain in culture.

2.5.4 Lentiviral Transduction of ADA$^-$ Skin Fibroblasts, Mesenchymal Stem Cells and B-Lymphoblastoid Cell Line

200,000 fibroblasts or MSCs were plated in a 6-well plate in 2mL complete DMEM, and 400,000 LCLs were likewise plated in complete RPMI. ADA or eGFP lentivirus were added at the appropriate MOI. The cells were then incubated at 37°C until analysed for transgene expression one week following the transduction (ADA staining protocol described in section 2.6.1). The cells were analysed for activity by Dr Lynette Fairbanks, Purine Research Laboratory, Guy's Hospital, London.

2.5.5 Lentiviral Transduction of Haematopoietic Stem cells

Mobilised peripheral blood derived CD34$^+$ cells were obtained from the Department of Haematology, UCL. The cells were counted and resuspended in transduction media to give a cell density of $1x10^6$ cells/mL (Refer to Table 2.2 for media composition). Lentivirus was added immediately at the required MOI and incubated with the cells for 24 hours. Following the transduction period, the cells were washed and resuspended in fresh transduction medium at a cell density of $1x10^6$ cells/mL. The cells were maintained in culture for 2-3 days, prior to being analysed for transgene expression by flow cytometry to determine the transduction efficiency (ADA staining protocol described in section 2.6.1).

2.6 FUNCTIONAL ASSAYS

2.6.1 Intracellular Flow Cytometry Assay

The primary anti-ADA monoclonal IgG1 antibody (IC5) was a generous gift from Prof. M.S. Hershfield (Department of Medicine, Duke University Medical Centre, North Carolina, USA). To carry out the intracellular staining for the ADA protein, the cells were fixed and permeabilised using the Intrastain Fixation and Permeabilisation Kit (DAKO, Glostrup, Denmark) using a three step staining

protocol. The cell samples (approximately $1x10^6$ cells for cell lines) were collected and washed in PBS before resuspending in 50μL PBS. 100μL Intrastain Reagent A (<6% formaldehyde -DAKO) was added to each sample to fix the cells, and was incubated for 15 minutes at room temperature. The cells were then washed with PBS and pelleted. The cell pellet was resuspended in 100μL Intrastain Reagent B (DAKO), containing saponin to permeabilise the cells. The appropriate volume of the appropriate antibody was then added and left to incubate for 20 minutes at room temperature. 5μL of 1mg/mL mouse anti-ADA or 10μL isotype control (PharMingen) were incubated with the cells. 1μL of the biotinylated anti-IgG1 (PharMingen) and streptavidin-phycoerythrin (SA-PE) (PharMingen) were then used. Following the final incubation with the SA-PE, the samples were washed and resuspended in PBS to be analysed in the flow cytometer. 10,000 cells were analysed based on forward scatter and side scatter values to exclude non-viable cells.

2.6.2 Western Blot

Approximately $1x10^6$ cells to be analysed were collected and lysed using lysis buffer (20mM Tris, pH8, 1% v/v Nonidet P-40, 0.13M NaCl, 10mM NaF, 1mM PMSF, Na_3VO_4, 1x leupeptin, 1x pepstatin, 1mM DTT). The samples were left on ice for 10 minutes and were then centrifuged at 12,000 x g for 1 minute at room temperature. The supernatant was added to an equal volume of loading buffer (0.125M TRIS, 14μM bromophenol blue, 2% SDS, 2% β-mercaptoethanol). The proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto a nitrocellulose membrane using a semi-dry transfer method at 12V for 20 minutes. The nitrocellulose membrane was incubated in 2.5% milk-PBS/0.1%Tween blocking solution for 30 minutes. The anti-ADA antibody (0.2μg/μl, Santa Cruz) was then added to the blocking buffer at a 1:100 dilution and was allowed to bind for 2 hours at room temperature. The blot was washed five times with PBS/0.1%Tween prior to the addition of the secondary antibody (Donkey anti-goat-HRP, Serotec) at a 1:1000 dilution. The HRP (horseradish peroxidase) labelled secondary antibody was detected by enhanced chemiluminescence (ECL - Amersham Pharmacia Biotech), and the protein was then visualised using Kodak BioMax film.

2.6.3 ADA Activity Assay

The enzymatic assay was carried out at the Purine Research Unit, Guy's Hospital, London, by Dr Lynette Fairbanks. ADA enzyme activity was established by the measurement of the concentration of inosine and hypoxanthine produced from adenosine (refer to Figure 1.2). The cells of interest were pelleted and washed twice in PBS. Cell lysates were then obtained by two rapid freeze-thaw cycles, and the cellular debris was removed by centrifugation. 25μL of the 500μL supernatant was added to 100μL 100mM phosphate buffer, pH 7.4 and 75μL 10mM adenosine. For the control sample, the reaction was immediately stopped by the addition of 50μL 40% trichloroacetic acid (TCA) to precipitate the protein. The other samples were incubated at 37°C for 30 minutes, after which the reaction was stopped by the addition of TCA. The samples were centrifuged at 10,000xg for 2 minutes. Water saturated ether was then added to the supernatant. The samples were vortexed and the ether layer was removed. This washing step was repeated 4 times. The samples were then loaded onto a hydrophobic/ionic high pressure liquid chromatography (HPLC), and eluted using tetrabutylammonium hydrogen sulphate (TBA) acetate (an ion pair agent). The reaction reagents and products were separated by HPLC and the values obtained were expressed as nmoles of inosine and hypoxanthine produced per hour per gram of total protein.

2.6.4 Protein Estimation by the Lowry Method

The samples used for the activity assay were analysed for total protein concentration using the Lowry method (Lowry *et al.*, 1951). The sample (25μL) was added to 1mL solution containing 2% Na_2CO_3 in 0.1M NaOH, 1% $CuSO_4.5H_20$, 2% Na/K tartrate and 1mg/mL BSA. The samples were allowed to incubate for 10 minutes at room temperature. 100μL Folin & Ciocalteau's reagent was then added and was left for 30 minutes. The absorbance was read at 740 nm, and the values obtained were extrapolated from a BSA standard curve to obtain the concentrations of the products.

2.6.5 Haematopoietic Colony Assay

Haematopoietic colony formation was analysed following the transduction of $CD34^+$ cells. 1000 cells were added to 500μL media, which was then mixed with 2.5 mL of methylcellulose (composed of 1% methylcellulose in Iscove's MDM, 30% foetal

bovine serum, 1% bovine serum albumin, 3U/mL recombinant human erythropoietin, 10^{-4} M 2-mercaptoethanol, 2mM L-glutamine, 50ng/mL recombinant human stem cell factor, 20ng/mL recombinant human GM-CSF, 20ng/mL recombinant human IL-3, 20ng/mL recombinant human IL-6, 20ng/mL recombinant human G-CSF – from Stem Cell Technologies). Approximately 1.5mL of the cells in methylcellulose was added carefully to a 35mm plate. The cells were incubated at 37°C, and were analysed for colony formation between days 10-14. Colony type was identified by morphology, and the number of colonies recorded using an inverted light microscope. Individual colonies were picked using a P20 Gilson pipette set on 4μL and added to 50μL lysis buffer (1xPCR buffer, 0.5% NP40, 0.5% Tween-20, 0.9mg/mL Proteinase K). The DNA was isolated from the colonies by incubation in lysis buffer at 60°C for 1 hour followed by 95°C for 5 minutes. 12.5μL was then used as a template for the PCR as described in the following section.

2.6.6 PCR Analysis

Genomic DNA was extracted from the cells using the Qiagen DNeasy kit according to manufacturers' instructions. Briefly, cell samples were lysed using Proteinase K, and the DNA was bound to the silica gel column. The column was washed to remove contaminants prior to DNA elution. PCR was performed using primers specific for the gammaretroviral backbone and the ADA transgene, amplifying a 310 base pair product. Each primer (Invitrogen) was 20 base pairs long, where the sequence of the forward primer was CAC CTT TAA CCG AGA CCT CA, and the reverse primer CTA CTT TGG GCT TGT CGA AG. The primers were used at 50pmoles per 50μL reaction, the magnesium chloride at 1mM concentration, with annealing temperature of 55°C, and 35 repeat cycles.

2.6.7 Real Time PCR Analysis

Genomic DNA was extracted from the cells using the Qiagen DNeasy kit as previously described. Primers and probe sequences for ADA cDNA were designed by Dr Klaus Kuehlcke (EUFETS, Germany) and amplified a product within the ADA gene. The primer (Invitrogen) and probe (MWG Biotech, Ebersberg, Germany) sequences were as follows:

ADA Forward: CCTGGCCAAGTTTGACTACTACATG

ADA Reverse: TCTCTACAAACTCATAGGCGATCCT
ADA Probe: FAM-CTATCGCGGGCTGCCGGGA-TAMRA.

β-actin was used as an internal control to determine the number of cells in each reaction, based on each cell having two copies of β-actin. Primers and probe sequences for human β-actin were designed by Steve Howe and Doug King (ICH, London) and were as follows:
β-actin forward: TCACCCACACTGTGCCCATCTACGA
β-actin reverse: CAGCGGAACCGCTCATTGCCAATGG
β-actin probe: VIC-ATGCCCTCCCCCATGCCA-TAMRA

Reaction conditions were as follows: 95°C for 10 minutes, then 40 repeat cycles of 95°C for 15 seconds and 60°C for 1 minute. The universal master mix was obtained from Applied Biosystems (CA, USA). The primers were used at a final concentration of 900nM and the probes at 5 μM. The PCR reactions were performed on an ABI Prism 7000 SDS (Applied Biosystems) real time thermocycler. The values obtained were compared to the ADA plasmid standard curve which ranged from 1 to 10,000 copies of the ADA gammaretroviral plasmid (Figure 2.1) to calculate absolute numbers of viral copies. These values were then compared with the β-actin DNA standard curve (Figure 2.2) generated by Steve Howe and Doug King (ICH, London) to estimate number of copies per cell.

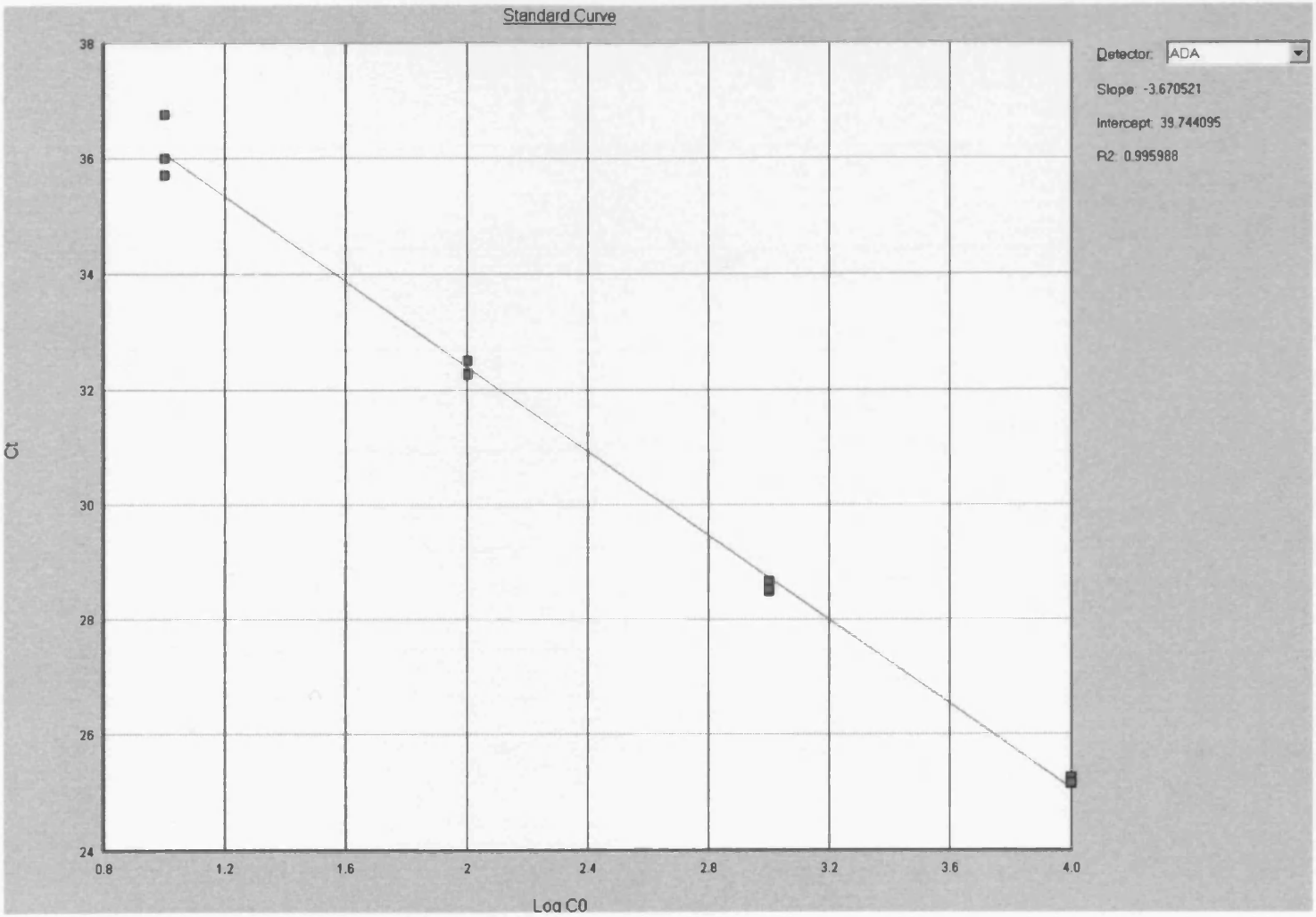


Figure 2.1. ADA standard curve. This standard curve was utilised to calculate the number of ADA cDNA copies in a sample. The r^2 value is 0.995.

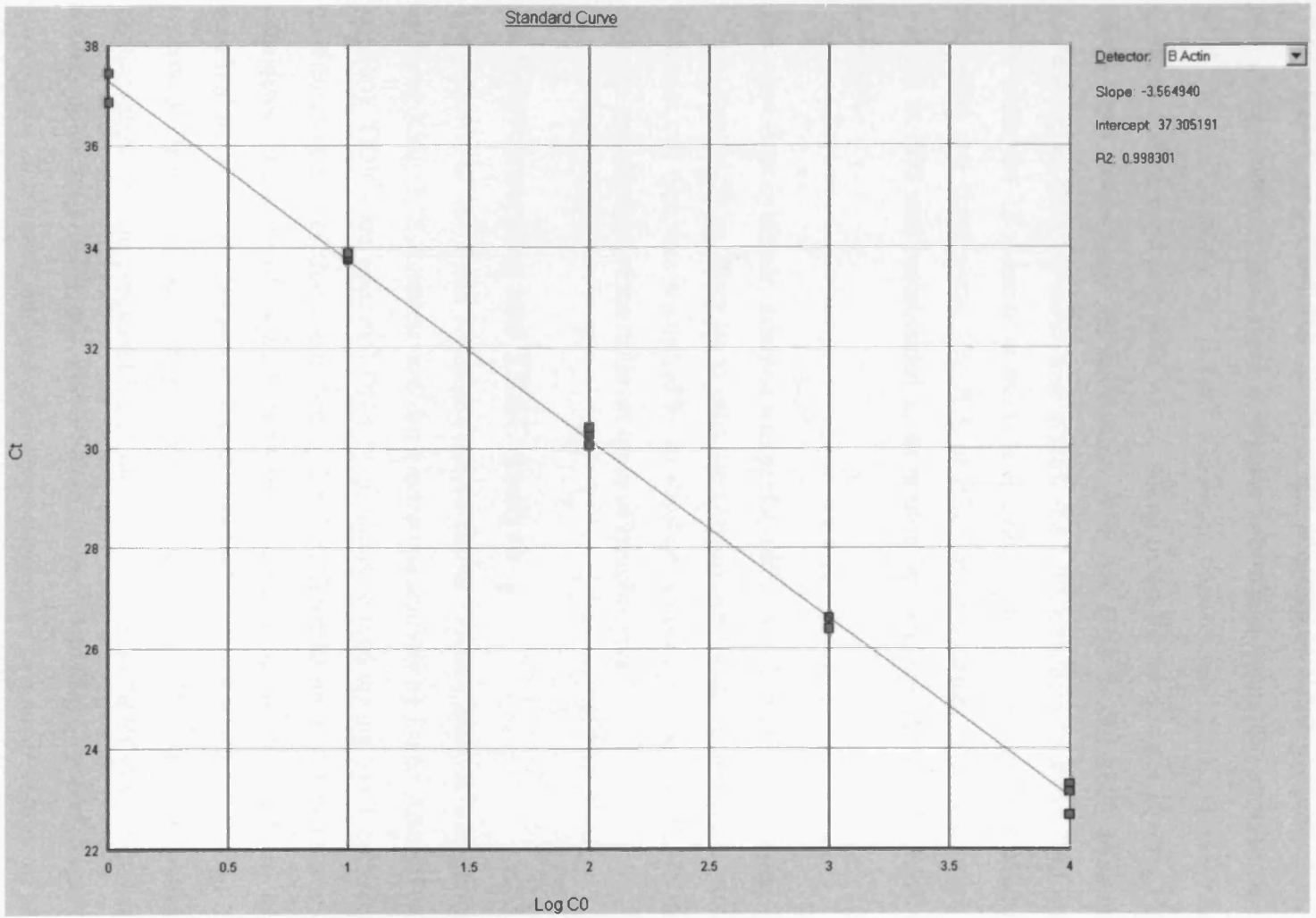


Figure 2.2. β -actin standard curve. This standard curve was utilised to calculate the number of ADA cDNA copies per cell in a sample. The r^2 value is 0.998.

2.6.8 Analysis of Patient Mononuclear Cells by Flow Cytometry

A three-colour flow cytometry analysis was performed on patient whole blood using antibodies to the following surface markers: CD3, CD4, CD8, CD16+CD56, CD19, CD27, CD45, and CD45RO (all from BD Biosciences listed in Table 2.3). This phenotype panel was performed at regular intervals to assess the response to PEG-ADA or gene therapy on patient leukocyte populations. 5 μL of the relevant antibodies were incubated with 100μL whole blood for 30 minutes at 4°C. The red blood cells were lysed by incubation with 1x lysis buffer (BD Biosciences, containing less than 5% diethylene glycol, and 1.5% paraformaldehyde (PFA)) made up in water for 15 minutes at room temperature. The samples were washed twice with PBS and fixed using 1% PFA in PBS for 15 minutes. The cells were then washed in PBS and resuspended in an appropriate volume of PBS/1% BSA to be analysed.

The same flow cytometry analysis was performed by the Clinical Immunology lab at Great Ormond Street Hospital to estimate lymphocyte counts. The percentage of the particular cell type was multiplied by an absolute lymphocyte count of CD45 cells to estimate the numbers of the different types of lymphocytes.

2.6.9 Spectratyping and TREC Analysis

The majority of the T cell receptor excision circles (TREC) analysis was performed by Doug King (ICH, London) and the spectratype analysis by Stuart Adams (GOSH, London). TRECs are episomal DNA by-products formed during the T cell receptor gene rearrangements that occur during T cell differentiation in the thymus and are considered markers of thymic activity. Spectratyping is an analysis of the distributions of CDR3 lengths to reveal whether there is a normal usage of T cell receptors. Briefly, blood samples were obtained from ADA deficient patients at regular intervals. The peripheral blood mononuclear cells (PBMCs) were isolated by density gradient (1.077g/mL Ficoll-Paque from Pharmacia), by centrifugation at 1,000xg for 20 minutes, and the cells were resuspended in a small volume of MACS buffer (Miltenyi Biotec, Cologne, Germany) to achieve approximately $5x10^4$ cells/μL. A proportion of the cells was pelleted and kept at -80°C for later TREC analysis. The remaining cells were selected for CD4 and CD8 expression using

Miltenyi magnetic cell sorting (Miltenyi Biotec). A proportion of the $CD4^+$ and $CD8^+$ cells were kept for TREC analysis. The remaining $CD4^+$ and $CD8^+$ cells were pelleted and resuspended in Tri Reagent for RNA extraction. Chloroform was added to the sample and mixed, and the samples were centrifuged at 10,000xg in the microcentrifuge. Isopropanol was added to the top aqueous phase and the samples were stored at -20°C to enhance the precipitation of RNA. The samples were centrifuged at 10,000xg in the microcentrifuge and the media was carefully removed. An ethanol wash was performed, 20μL water was added and the samples were incubated at 60°C to facilitate the sample resuspension. dNTPs, oligo $d(T)_{16}$ (Roche) and RNase inhibitor was added to each tube of RNA. The samples were incubated at 70°C for 15 minutes, after which $MgCl_2$ PCR buffer (Promega) and reverse transcriptase was added to each sample and incubated at 42°C for 1 hour. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. Following the first PCR reaction, a run-off PCR was performed and the lengths of the CDR3 regions were determined using a MegaBase sequencer machine.

For the samples analysed for TREC activity, the cell pellets were resuspended in buffer containing Tween-20, proteinase K and NP40 and incubated at 56°C for 2 hours. The samples were centrifuged at 10,000xg in the microcentrifuge, and the proteinase K was inactivated by incubating the samples at 95°C for 15 minutes. A small sample of the extracted DNA was then used for real time PCR analysis. The primers and probes were as follows:

Forward CACATCCCTTTCAACCATGCT

Reverse GCCAGCTGCAGGGTTTAGG

Probe FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA

Reaction conditions were as previously described in section 2.6.7. The primers were used at a final concentration of 300nM and the probe at 100 nM. For each run, a standard curve was generated from triplicate samples of 5-fold serially diluted known copies of plasmid DNA containing a TREC fragment. These values were then compared with the β-actin DNA standard curve as previously described to estimate number of copies per cell.

3

ANALYSIS OF PEG-ADA TREATMENT FOR ADA DEFICIENCY

3.1 INTRODUCTION

Treatment options for patients with ADA-SCID are limited. The therapy of choice is bone marrow transplantation if a fully matched donor is available. The ideal donor is a fully matched sibling, however a matched unrelated donor (MUD) is also commonly used. In the presence of a matched donor, the prognosis is good, as this type of transplant usually leads to complete immune reconstitution. However, this is usually only available for approximately a third of the patients, as many do not find a fully matched related or unrelated donor. Hence, if transplant is not an option, the patient can be treated with PEG-ADA enzyme replacement therapy. The treatment involves weekly or bi-weekly intramuscular injections of bovine ADA which has been conjugated to PEG in order to increase the half-life of the enzyme. For many patients, PEG-ADA therapy is often successful. However, although patients usually initially respond well to PEG-ADA, for some patients this response can deteriorate as they are maintained on this treatment for a long period of time and may also mount an immune response against the bovine ADA (reviewed by Hershfield, 1995 and by Hershfield & Mitchell, 2001). No formal data has been previously available on the immune recovery of patients during PEG-ADA therapy.

We looked in detail at the immune recovery during PEG-ADA therapy in three ADA-SCID patients. A number of assays were performed to analyse the patients' progress: measurements of lymphocyte counts (performed by the Clinical Laboratory at Great Ormond Street Hospital) and dATP concentrations (performed by Lynette Fairbanks at the Purine Research Laboratory, Guys' Hospital), analysis of the production of naïve immune cells by flow cytometry, spectratyping (performed with the help of Stuart Adams, ICH) and TREC analyses (performed with the help of Doug King, ICH).

3.2 RESULTS

3.2.1 Analysis of Lymphocyte Counts

Patient 1 is a boy of 3 years old who has been treated with PEG-ADA for nearly 3 years. Patient 2, a one-year old girl, was diagnosed with ADA-SCID at 2 months of age following recurrent infections and commenced PEG-ADA at 3 months of age. Patient 3, a boy of 4 years old, was treated with PEG-ADA for more than 3 years prior to being treated with gene therapy. Blood samples were taken from each patient at regular intervals to assess lymphocyte reconstitution. Prior to PEG-ADA all three patients had nearly undetectable lymphocyte counts as expected for ADA deficient patients. Patients 1 and 2 responded well to PEG-ADA therapy, with their lymphocyte counts increasing following initiation of the treatment and remaining at adequate levels (approximately 1,200 and 1,900 cells/μL respectively) for over 2 years and up to 1 year of treatment respectively (Figures 3.1A and B). Their lymphocyte counts are now at levels just below that of normal age matched controls (see Appendix 1). However, although the absolute lymphocyte counts were near normal, it was apparent that the number of $CD4^+$ cells was unusually low and a reverse CD4/CD8 ratio was observed. Therefore, although the two patients displayed good immune recovery, it was not entirely normal. Measurements of dATP levels have revealed that patients 1 and 2 both remain detoxified (data not shown), and both are clinically free of infection and thriving. Patient 3 did not respond as well as patients 1 and 2 to the PEG-ADA treatment as demonstrated by his low lymphocyte counts (Figure 3.1C). Following the start of PEG-ADA his absolute lymphocyte counts increased, however this was not sustained but decreased rapidly to approximately 600 cells/μL. This was in spite of achieving good detoxification as demonstrated by the low levels of dATP (see Figure 5.5). It was therefore clear that his lymphocyte counts, in particular the T cell counts, were well below normal and the patient showed a poor response to PEG-ADA treatment.

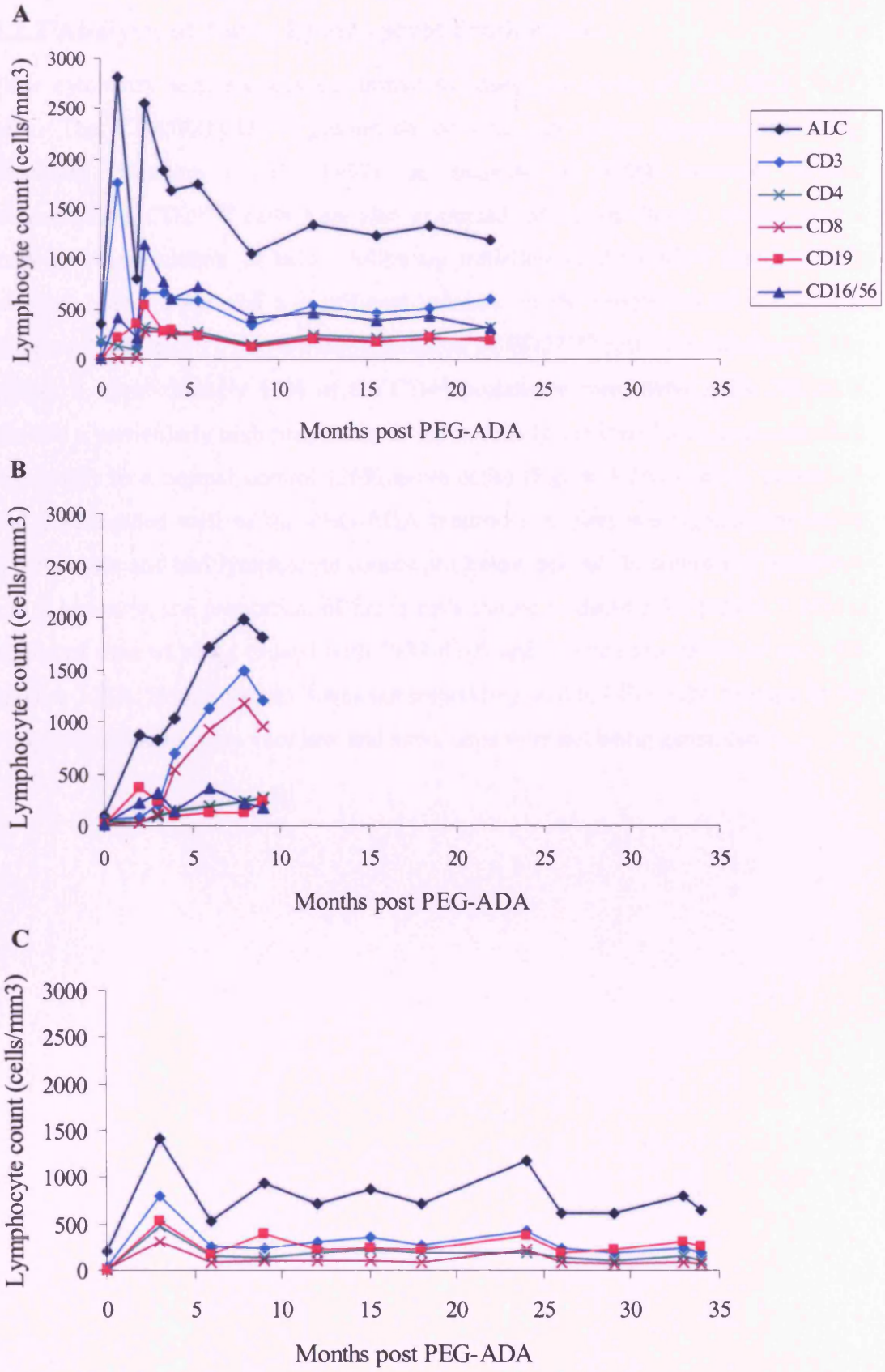


Figure 3.1. Lymphocyte counts during PEG-ADA treatment. Blood samples were taken at regular intervals and flow cytometry analysis was performed to estimate the number of cells per μL of the lymphocyte subsets.

A, Patient 1; **B,** Patient 2; **C,** Patient 3.

3.2.2 Analysis of Naïve Lymphocyte Production

Flow cytometry analysis was performed to assess the levels of $CD45RO^{-}CD27^{+}$ cells. The $CD45RO^{-}CD27^{+}$ proportion of cells are naïve lymphocytes being generated (Hamann *et al.*, 1997), an increase of which indicates thymic reconstitution. $CD27^{high}$ cells were also examined, which are thought to represent a truly naïve population of cells. Following initiation of PEG-ADA therapy, both patients 1 and 2 showed a significant increase in the proportion of naïve cells (Figures 3.2 B and C), and a clear population of $CD27^{high}$ cells also developed. For patient 2, approximately 10% of the CD45 population were naïve cells. Patient 1 showed a particularly high proportion of naïve cells (more than 30%), and compared favourably to a normal control (36% naïve cells) (Figure 3.2A). Hence, patients 1 and 2 responded well to the PEG-ADA treatment, as they were generating naïve lymphocytes and had lymphocyte counts just below normal. In contrast to patients 1 and 2 however, the proportion of naïve cells started to decline for patient 3 after a period of time of being treated with PEG-ADA and reached low levels of only 4% (Figure 3.2D). Hence, patient 3 was not responding well to PEG-ADA therapy as the lymphocyte counts were very low and naïve cells were not being generated.

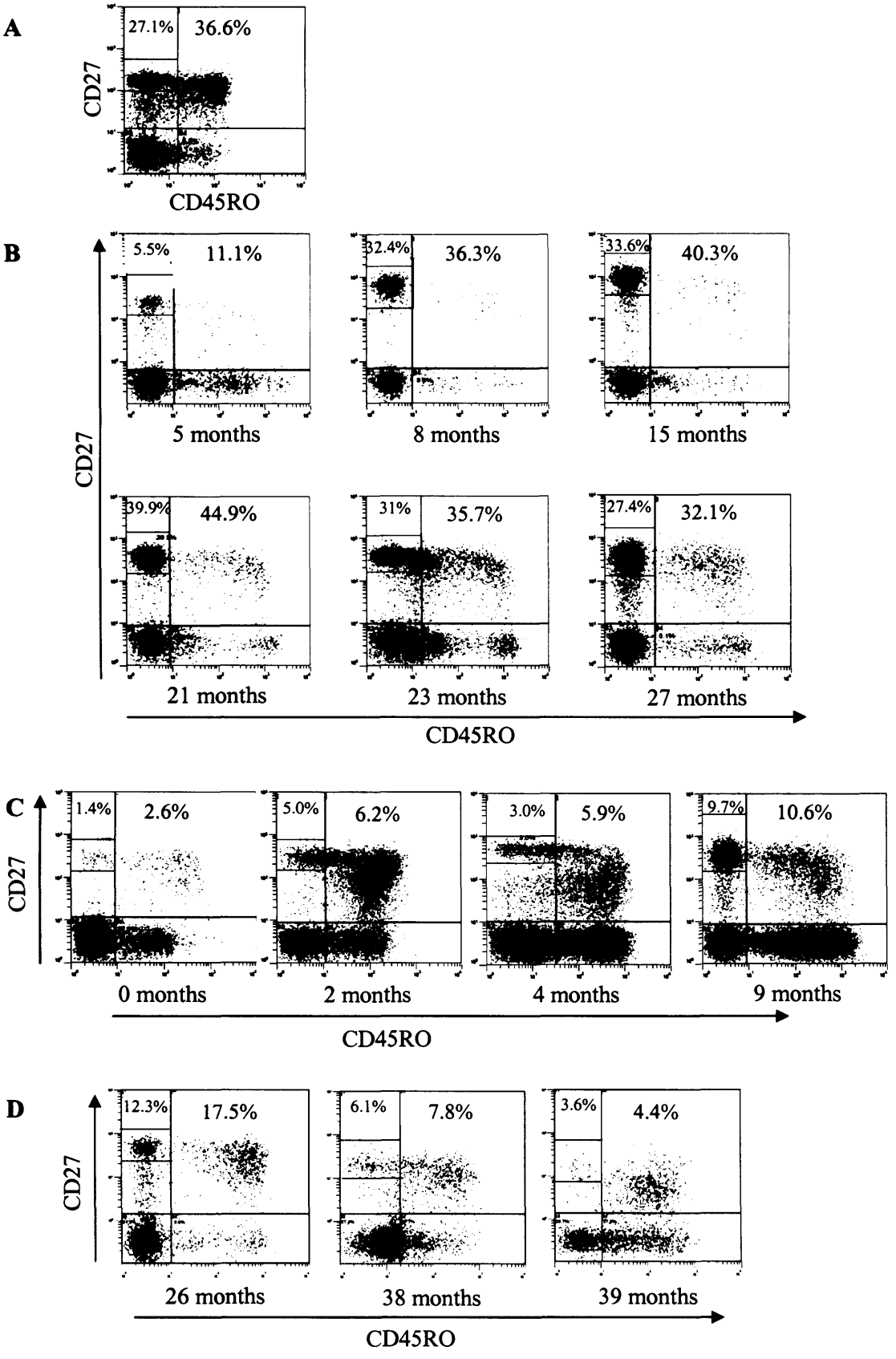


Figure 3.2. Determination of the proportion of naïve lymphocytes. Blood samples were regularly analysed by flow cytometry. The CD45RO$^-$CD27$^+$ population of cells represents naïve lymphocytes being produced (percentage of which is written in the larger font). A separate gate has been drawn for cells expressing high levels of CD27 (percentage of which is written in the smaller font).

A, Normal control; **B,** Patient 1; **C,** Patient 2; **D,** Patient 3.

3.2.3 Analysis of Thymic Function

Thymic function was evaluated by assessing the production of T cell receptor excision circles (TRECs) in the $CD4^+$ and $CD8^+$ subsets of T cells. TRECs are episomal DNA by-products formed during the T cell receptor gene rearrangements that occur during T cell differentiation in the thymus. As the TRECs are episomal, they do not replicate during mitosis and are therefore diluted as a result of cell proliferation. Hence, the detection of TRECs in peripheral blood is considered a marker of thymic activity. Prior to PEG-ADA, no or only very low TREC activity was detectable in the three ADA deficient patients. As a result of PEG-ADA therapy, TREC activity increased dramatically in patients 1 and 2 in both the $CD4^+$ and $CD8^+$ T cell populations (Figure 3.3). Talvensaari *et al.* (2002) reported TREC values of approximately 4000 TRECs per μg $CD3^+$ DNA from healthy young donors. Patient 1 was found to have approximately 21,000 TRECs per 10^6 $CD3^+$ cells ($CD4^+$ and $CD8^+$ cells combined) and patient 2 had approximately 19,000 TRECs per 10^6 $CD3^+$ cells. Based on the estimate of 6pg DNA per diploid cell (Jeffreys *et al.*, 1988), 10^6 cells (which our TREC values are expressed as a function of) would contain approximately 6μg of DNA. Hence, the TREC values obtained equate to approximately 3,400 and 3,100 TRECs per μg $CD3^+$ DNA for patients 1 and 2 respectively. Therefore, these values were comparable to the TREC values observed in healthy young donors (Talvensaari *et al.*, 2002). This increase in TREC activity indicated that the thymic function of patients 1 and 2 had improved greatly as a result of the PEG-ADA treatment. Patient 3, however, showed no TREC activity during his PEG-ADA therapy (data not shown), confirming that immune reconstitution for patient 3 was poor.

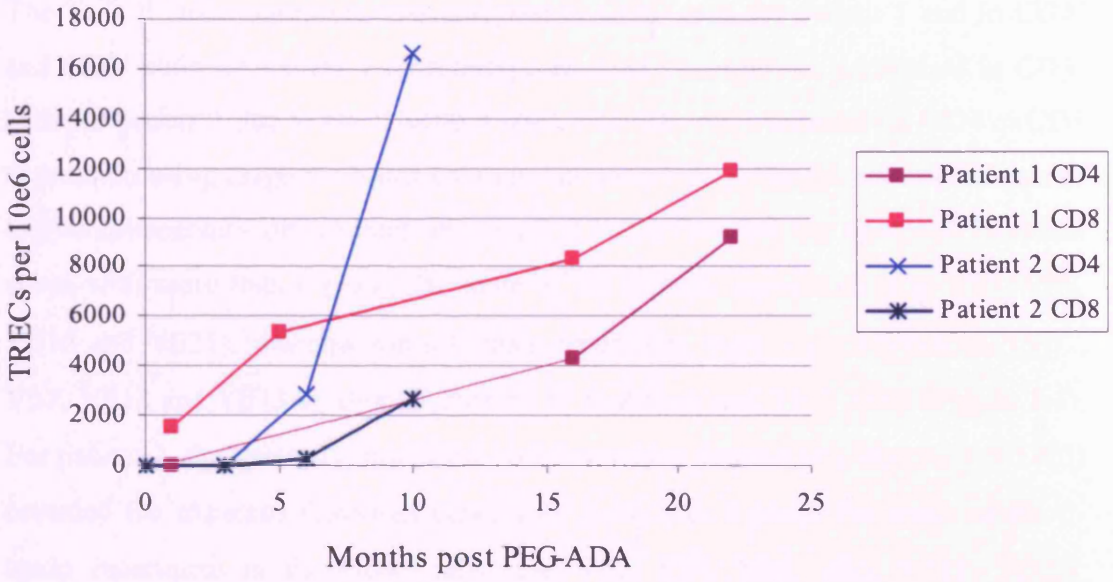


Figure 3.3. TREC values for patients 1 and 2 during PEG-ADA treatment. Patient blood samples were collected at regular intervals and $CD4^+$ and $CD8^+$ T cells were separated using magnetic beads column. Real-time PCR analysis was performed on the genomic DNA to estimate the numbers of TRECs in the samples.

3.2.4 Analysis of T Cell Receptor (TCR) Diversity

The TCR β-chain repertoire was analysed in $CD8^+$ cells for patient 1 and in $CD4^+$ and $CD8^+$ cells for patient 2 (spectratype analysis could not be performed in $CD4^+$ cells for patient 1 due to small sample size). The cells were selected for CD4 or CD8 expression using magnetic beads column. For patient 1, the length pattern analysis of the complementary determining regions 3 (CDR3) revealed the expected Gaussian curve with more than 6 peaks for some of the β-chain repertoires (e.g. Vβ5, Vβ9, Vβ16 and Vβ21), whereas some β-chain repertoires were more oligoclonal (Vβ1, Vβ2, Vβ12 and Vβ13A) after 27 months of treatment with PEG-ADA (Figure 3.4). For patient 2, the spectratyping of the complementary determining regions 3 (CDR3) revealed the expected Gaussian curve with more than 6 peaks for some of the β-chain repertoires in the $CD4^+$ cells (e.g. Vβ2, Vβ3, Vβ4, Vβ5, Vβ21, Vβ22), however some β-chain repertoires in the $CD8^+$ cells were more clonal (Vβ11, Vβ13B, Vβ16 and Vβ24) after 11 months of treatment with PEG-ADA (Figure 3.5), perhaps indicating prior antigen stimulation. Therefore, both patients 1 and 2 display near normal distributions of CDR3 lengths. Spectratype analysis was not performed for patient 3 during the PEG-ADA treatment.

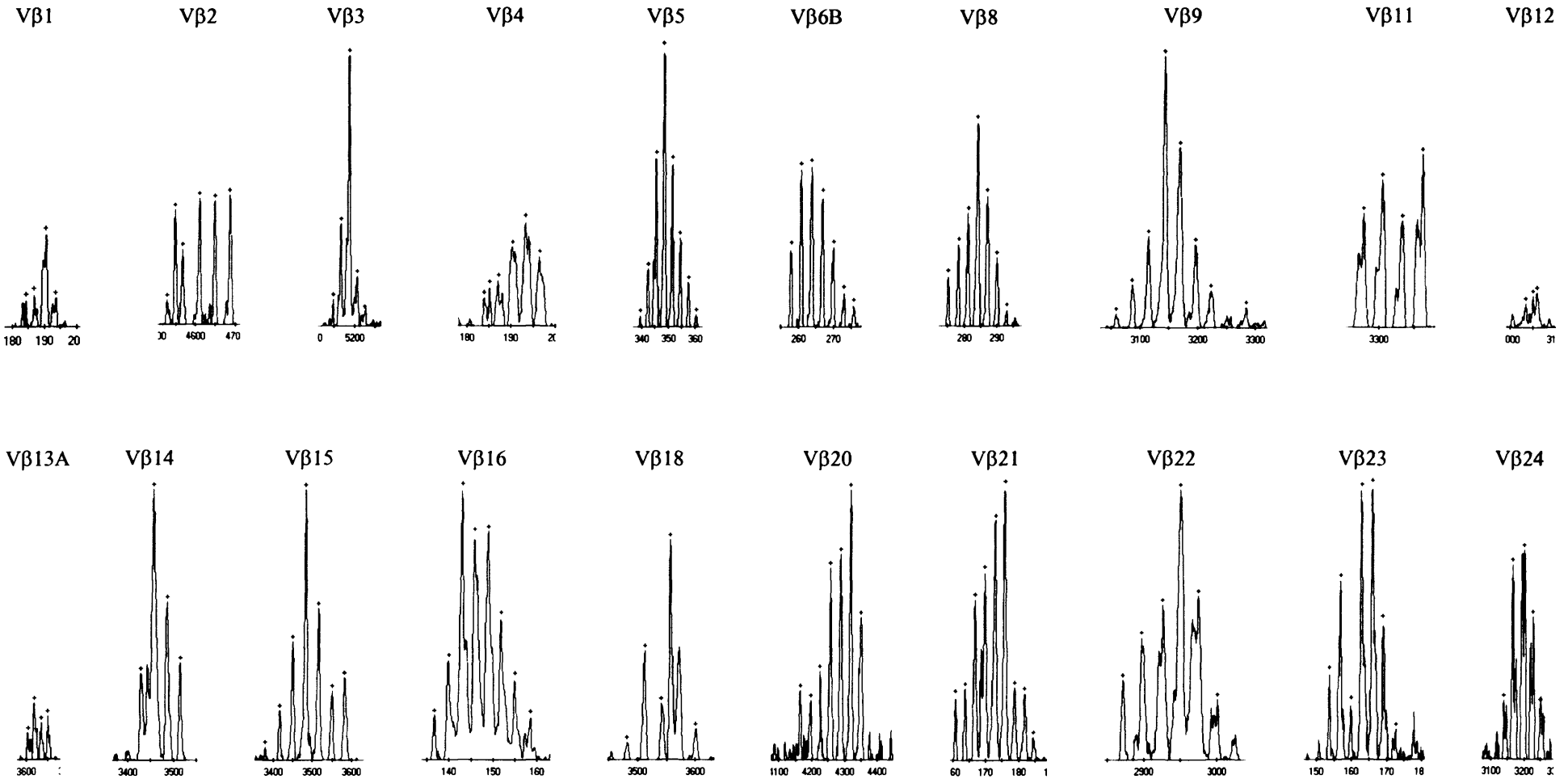


Figure 3.4. CD8 spectratypes for patient 1 after 27 months of treatment with PEG-ADA. Patient blood samples were collected at regular intervals and $CD8^+$ cellss were separated using magnetic beads columns. The RNA was extracted and reverse transcribed. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. A run-off PCR was then performed and the CDR3 lengths were determined using a MegaBase sequencer machine.

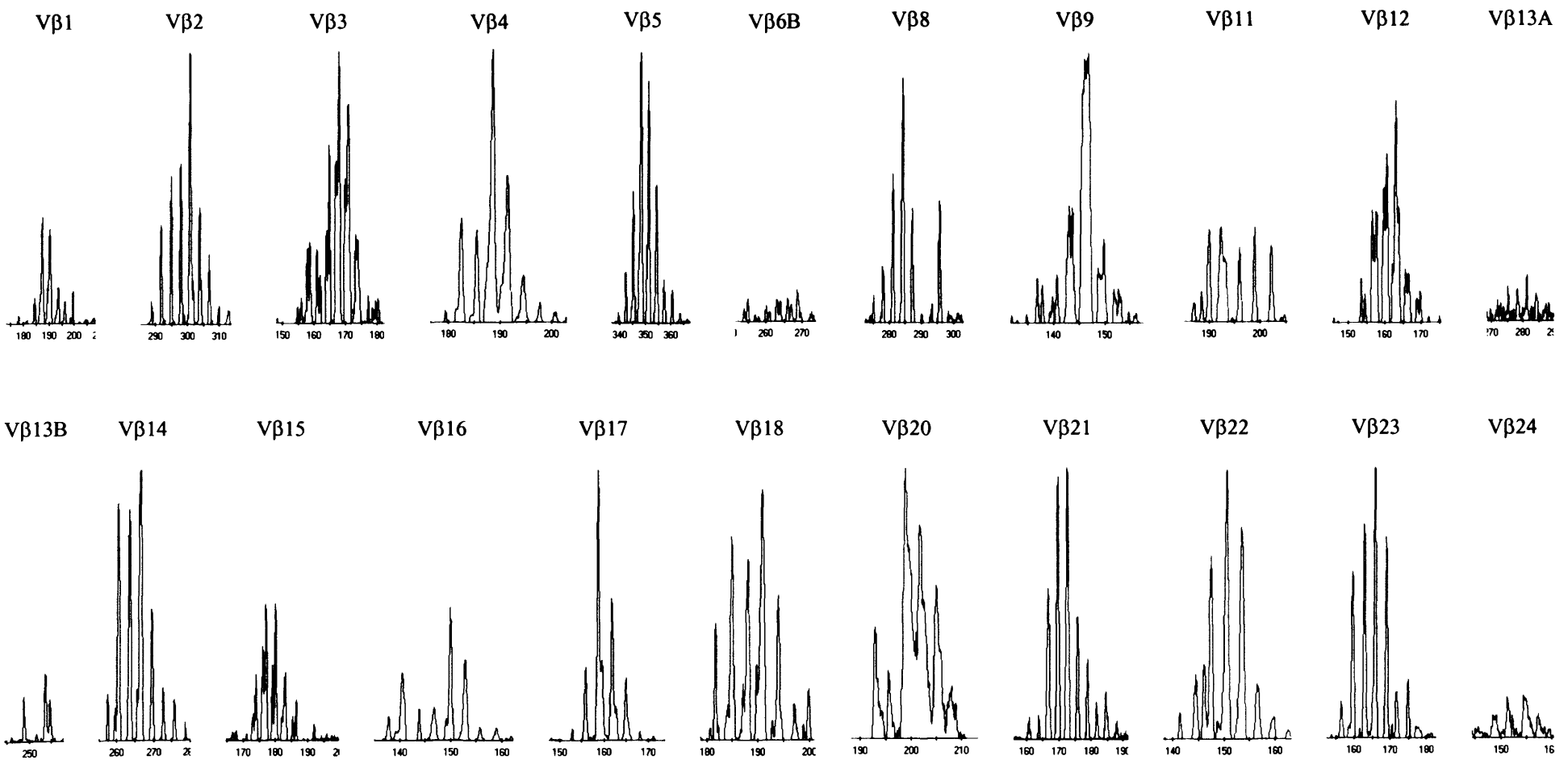


Figure 3.5A. CD4 spectratypes for patient 2 after 11 months of treatment with PEG-ADA. Patient blood samples were collected at regular intervals and $CD4^+$ cells were separated using magnetic beads columns. The RNA was extracted and reverse transcribed. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. A run-off PCR was then performed and the CDR3 lengths were determined using a MegaBase sequencer machine.

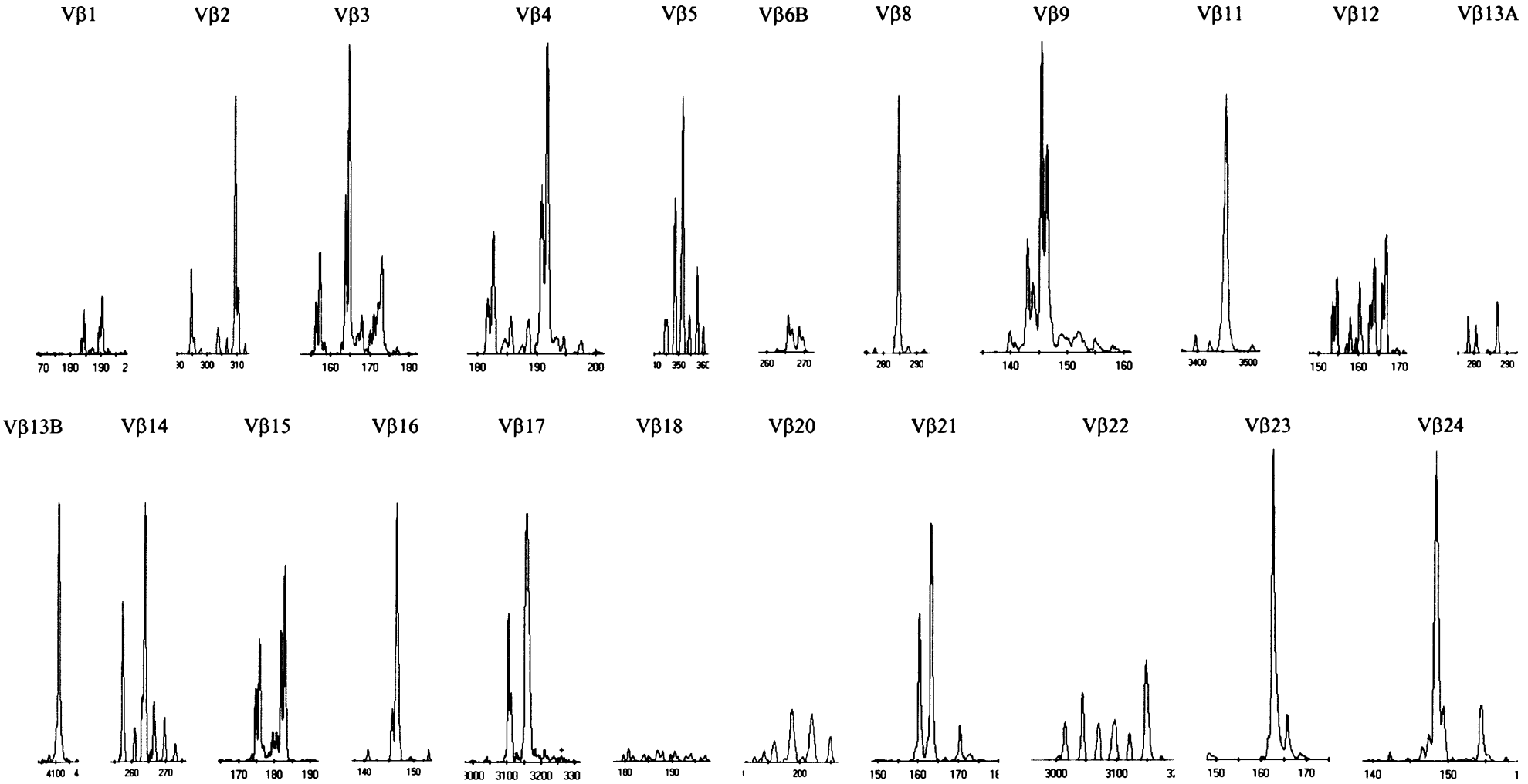


Figure 3.5B. CD8 spectratypes for patient 2 after 11 months of treatment with PEG-ADA. Patient blood samples were collected at regular intervals and $CD8^+$ cells were separated using magnetic beads columns. The RNA was extracted and reverse transcribed. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. A run-off PCR was then performed and the CDR3 lengths were determined using a MegaBase sequencer machine.

3.3 DISCUSSION

The response of three ADA-SCID patients to PEG-ADA therapy was analysed by several different methods to investigate their levels of immune reconstitution. Patient 1 has been treated with PEG-ADA for over 2 years, and patient 2 for nearly 1 year, both with some success. However, following three years of PEG-ADA treatment, the immune recovery of patient 3 was deteriorating.

Prior to PEG-ADA, all three patients had an almost undetectable lymphocyte count as is to be expected in ADA deficient patients who display a selective toxicity to B, T and NK cells. As a result of the detoxification incurred by the PEG-ADA treatment, the lymphocyte counts of patients 1 and 2 increased and stabilised at levels slightly below normal, suggesting a good immune recovery in these patients. Of the three patients analysed in this study, patient 2 appeared to do particularly well as her ALC was the highest. However, although patients 1 and 2 had reasonable lymphocyte counts, immune recovery was clearly not normal as indicated by the low CD4 counts and reverse CD4/CD8 ratios. Patient 3 had very low lymphocyte counts on diagnosis, which increased as a result of the enzyme replacement therapy. However, after 3 years of PEG-ADA therapy his ALC was very low, about half that of patient 1 and a third of the ALC of patient 2, suggesting that he had a poor immune recovery.

Analysis of expression of the markers CD45RO and CD27 can reveal the generation of new lymphocytes, as $CD45RO^-CD27^+$ cells represent a naïve cell population (Hamann *et al.*, 1997). Patients 1 and 2 showed a significant increase in the proportion of $CD45RO^-CD27^+$ cells as a result of PEG-ADA therapy, suggesting the production of naïve lymphocytes. This was further confirmed by the analysis of TRECs, which estimates the level of thymic function. Patients 1 and 2 showed no or very low thymic activity prior to PEG-ADA. However, following initiation of PEG-ADA treatment, the values of TRECs increased dramatically in both $CD4^+$ and $CD8^+$ cells. Thus naïve cells were being generated due to the detoxification by PEG-ADA. There is generally expected to be a clear correlation between the increase in naïve T cells according to the flow cytometry analysis and TREC analysis. Thus patient 1 would be expected to have higher TREC values than patient 2 as he had a higher proportion of $CD45RO^-CD27^+$ cells. However, this apparent discrepancy could be

due to a number of reasons, as TREC values are affected by the age of the individual, peripheral T lymphocyte replication leading to a dilution of TRECs, and the longetivity of T cells which can survive in the peripheral blood for between over 7 years (Talvensaari *et al.*, 2002). However, unlike patients 1 and 2, Patient 3 showed a decrease in the proportion of $CD45RO^{-}CD27^{+}$ cells following 3 years of PEG-ADA therapy. Moreover, throughout the PEG-ADA treatment no TREC activity could be detected in his $CD4^{+}$ or $CD8^{+}$ cells. Thus, he was not generating naïve lymphocytes in spite of the PEG-ADA treatment.

Spectratype analysis for patient 1 in $CD8^{+}$ cells and for patient 2 in $CD4^{+}$ and $CD8^{+}$ cells, revealed near normal Gaussian distributions for some β-chain repertoires. However, certain repertoires in the $CD8^{+}$ cells of patient 2 were more clonal, perhaps indicating an as yet incompletely formed repertoire (Talvensaari *et al.*, 2002) or prior antigen stimulation. The TCR polyclonality in most β-chain repertoires for patients 1 and 2 correlated with the high proportion of naïve cells and high TREC values.

Hence, patients 1 and 2 have responded well to the PEG-ADA therapy. They are both currently at home and are clinically well, and remain on prophylactic medication including immunoglobulin replacement. Both patients have showed an increase in their lymphocyte counts and thymic function is improving in both patients as evidenced by the increase in naïve T cells and the increased TREC activity. Moreover, both patients display a near normal repertoire of Vβ subfamilies. However, patient 3 has not responded as well to PEG-ADA therapy as evidenced by his low lymphocyte counts and decrease in the production of naïve lymphocytes. To our knowledge, this is the first time that the effects of PEG-ADA have been analysed in relation to thymic activity.

Previous studies of PEG-ADA therapy have reported good survival rates of nearly 80% (reviewed by Hershfield, 1995). Upon initiation of PEG-ADA treatment, a rapid decrease in toxic metabolites is usually observed to levels only slightly above normal (Hirschhorn *et al.*, 1980 & 1981; Hershfield, 1995). As a result of this detoxification, B cell numbers increase during the first month, followed by an increase in T cells at about 6-12 weeks of treatment. Approximately half of the patients who receive PEG-

ADA discontinue intravenous immunoglobulin (IVIG), and the level of immune function achieved has been sufficient to protect against opportunistic infections in most patients (Hershfield, 1995). The proliferative response of lymphocytes to mitogens also usually increases during the first two months and a thymic shadow may reappear (Hershfield *et al.*, 1993). However, as was observed with the patients in the current study, lymphocyte counts do not commonly reach normal levels of age-matched controls, and the degree of immune reconstitution can be varied. Of the patients treated previously with PEG-ADA, 50% have developed antibodies against the bovine ADA. This could potentially reduce the effectiveness of the treatment, however this has only been reported in a few patients. Although unusual, immune dysregulation may also occur as a result of PEG-ADA, including refractory immune haemolytic anaemia as was observed in two patients (Hershfield *et al.*, 1993). Nevertheless, PEG-ADA has been found to be a successful treatment for most ADA deficient children, and patients 1 and 2 have shown a similar immune recovery to what has been previously reported (Hershfield *et al.*, 1987; 1993 and 1995).

Hence it has been demonstrated that in the absence of a matched bone marrow transplant, PEG-ADA therapy can be a successful treatment for some patients, such as for patients 1 and 2. However, for some patients, like patient 3, PEG-ADA is ineffective and alternative treatments should be considered. Therefore, gene therapy was investigated as a potential treatment for patient 3, and experiments were performed in order to develop an effective gammaretroviral vector and gene therapy protocol.

4

CONSTRUCTION AND ASSESSMENT OF ADA GAMMARETROVIRAL VECTOR

4.1 INTRODUCTION

Gene therapy is a possible curative treatment for ADA deficiency. For gene therapy to be successful, it is thought that a high expression of ADA in the patient target cells is required to detoxify the system. Haematopoietic stem cells are used as cell delivery vehicles because they are able to repopulate the entire haematopoietic system (Hay, 1966) and should therefore deliver ADA expression to different haematopoietic cells. Viral vectors and the transduction protocol utilised therefore need to be optimised for efficient gene transfer to HSCs which should allow for a high expression of ADA.

Gammaretroviruses are the vectors of choice in current gene therapy trials for immunodeficiency as they efficiently integrate their genetic material into the host cell's genomic DNA, resulting in the long-term expression of the transgene. However, the requirement for efficient integration of the transgene to achieve good transgene expression needs to be balanced against the fact that high levels of integration can also lead to insertional mutagenesis, which will be discussed further in Chapter 8. Retroviral vectors have several other advantages as gene delivery vehicles, including a large packaging capacity of up to 10 kilobases, and the absence of vector-induced cellular immune responses as they do not encode viral proteins.

The gammaretrovirus used in our study is based on a vector constructed by Baum *et al.* in 1995, and includes the 3' LTR from the Spleen Focus Forming Virus (SFFV) and the 5'LTR derived from MESV (Murine Embryonic Stem Cell Virus). SFFV is a replication-defective form of the erythroblastic Friend Murine Leukaemia Virus, and has a U3 region encoding strong regulatory influences on cells of the myeloid lineage. MESV was generated by combining the LTR from PCMV (PCC4-cell-passaged Murine Sarcoma Virus) with a mutated primer binding site from an endogenous murine retrovirus and is known to resist silencing (Grez *et al*, 1990). A major concern associated with viral vectors is the phenomenon of silencing of LTR directed transcription with time. Silencing, a form of host defence mechanism, can be due to methylation dependent or independent molecular modifications, that result in promoter shut-off (Challita & Kohn, 1994; Bestor, 2000; Pannell *et al.*, 2000, Pannell & Ellis, 2001). It is therefore important to find a promoter for use in gene therapy

vectors which can resist this silencing process. Modification of the U3 region of the LTR and the primer binding sites, important regions for determining transcriptional activity, has been used to reduce the risk of silencing and to direct tissue tropism (Grez *et al*, 1990). The transgene expression in our viral vector is therefore regulated by the SFFV promoter, which has been shown to result in high transgene expression levels in all haematopoietic lineages (Baum *et al.*, 1995, 1997; Flasshove *et al.*, 2000).

The viral glycolipid membrane envelope determines the cell tropism of the vector. The viral vector can be encapsidated either by the envelope protein of that virus or that of a different virus (pseudotyping). The gammaretroviral vector used in this study was pseudotyped with the Gibbon Ape Leukaemia Virus (GaLV) envelope, provided by the PG13 packaging cell line, which has previously been reported to result in high gene transfer efficiencies into haematopoietic stem cells (Kiem *et al.*, 1997) and T cells (Lam *et al.*, 1996). The receptor for the GaLV envelope, glvr-1 (Pit-1), is a phosphate transport protein (Johann *et al.*, 1992) expressed on several different cell types. The GaLV envelope confers the ability to infect human, rat, cat, dog and monkey cells, but importantly not murine cells. Thus, it alters the tropism of the murine gammaretrovirus to allow the infection of human cells. At the same time, pseudotyping with the GaLV envelope reduces the risk of re-infection of the PG13s, leading to decreased possibilities of recombination events and the production of replication competent retroviruses.

The Woodchuck Post-transcriptional Regulatory Element (WPRE) was included in the ADA-gammaretrovirus as it has been shown to enhance the expression levels of the transgene eight-fold post-transcriptionally (Zufferey *et al.*, 1999). It is an element derived from the Woodchuck hepatitis virus (Donello *et al.*, 1998) and is thought to function by affecting polyadenylation and promoting the nuclear export of vector RNA (Huang, J. & Liang, 1993; Huang, Z.M. & Yen, 1994; Zufferey *et al.*, 1999). Zufferey *et al.* importantly showed that the WPRE is not reliant on the type of vector, transgene, or promoter. Nor is it dependant on the cycling status of the cell, the cell type or species. Increased ADA expression is sought-after as high levels of ADA may be required to remove the toxic metabolites in the cells of ADA deficient patients. Importantly, high levels of ADA expression are thought not to be toxic as

PEG-ADA therapy results in very high extracellular ADA activity, and individuals with ADA over-expression intracellularly have been found to be healthy. Recent concerns regarding the safety of WPRE are discussed in chapter 8.

The following experiments were therefore carried out to generate an efficient gammaretroviral vector to be used in gene therapy. This vector was tested on different patient cells to evaluate its transduction efficiency and to determine whether it had any toxic effects on the cells. Once this had been verified, a protocol for the efficient transduction of $CD34^+$ cells was established.

4.2 RESULTS

4.2.1 Construction of the ADA Gammaretroviral Vector

The ADA cDNA was generated by PCR from the pOTB7 vector carrying the ADA (kind gift from Prof D. Valerio, Department of Gene Therapy, University of Leiden, Netherlands), using primers specifically designed to amplify the ADA cDNA and include *XbaI* linkers (Figure 4.1A). These restriction sites were then utilised to insert the ADA into the intermediate cloning vector pBlueScript (pBS) (Figure 4.1B). This step facilitated the cloning procedure as the vector has several restriction sites.

The gammaretroviral vector (SFada/W) was constructed by removing the ADA-WPRE fragment by an *XbaI/XhoI* digest from the pBS, and ligating it into the gammaretroviral vector already containing the SFFV promoter. Therefore, the gammaretroviral vector carried the SFFV promoter, the ADA transgene and the WPRE element (Figure 4.1C). The correct design of the constructed vector was confirmed by restriction digest patterns (Figure 4.1D), and sequencing between the two LTRs (data not shown).

The gammaretroviral eGFP vector (from the Institute of Child Health Molecular Immunology plasmid library) was utilized in some experiments as a control vector (Figure 4.1E). Although this vector did not incorporate the WPRE element, a high level of eGFP expression was observed.

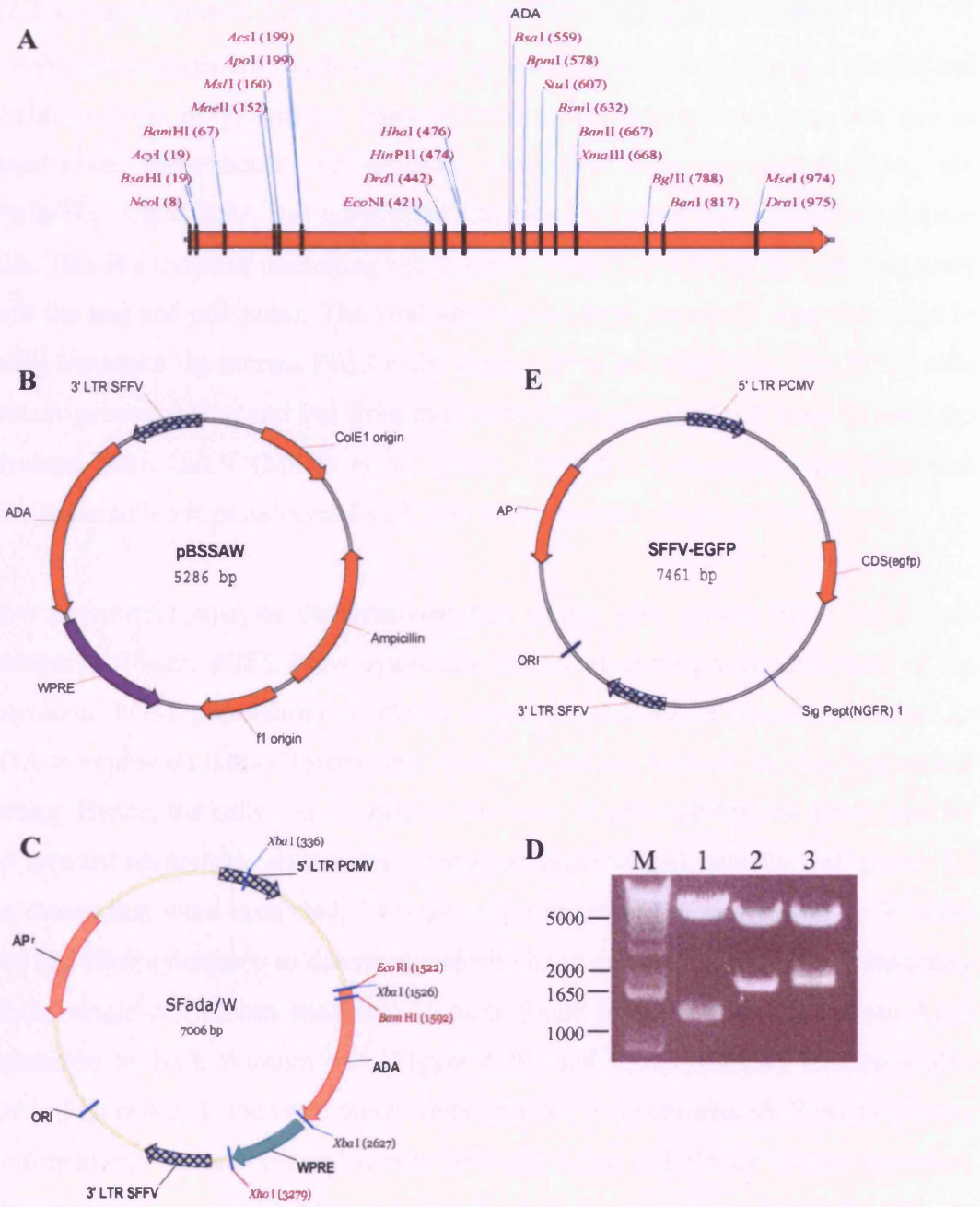


Figure 4.1. Generation of gammaretroviral vectors.

A, Map of the human ADA cDNA. The ADA transgene was generated by PCR to incorporate *XbaI* linkers, which were then used to incorporate the transgene into the gammaretroviral and lentiviral vectors. **B,** Vector map of pBlueScript SFFV-ADA-WPRE (SAW). This vector was used as an intermediate cloning vector to facilitate the introduction of the ADA-WPRE fragment into the gammaretroviral vector, and the SAW fragment into the lentiviral vector as described in chapter 6. **C,** Vector map of the ADA-gammaretroviral construct (SFada/W). The ADA-WPRE fragment was digested from the pBS-SAW by an *XbaI*/*XhoI* digest and was then ligated into the gammaretroviral vector. **D,** Restriction digest gel to confirm correct design of SFada/W. M, marker; 1, *XbaI* digest (*XbaI* does not cut at position 2627 probably because of methylation); 2, *BamHI*/*XhoI* digest; 3, *EcoRI*/*XhoI* digest. **E,** Vector map of the gammaretroviral GFP vector. The vector encodes eGFP, also under the control of the SFFV.

4.2.2 Generation of Gammaretroviral Packaging Cell Lines

A stable gammaretroviral packaging cell line was generated as this is a simple and reliable method of generating gammaretrovirus of reproducible titres for use in transduction experiments. To generate gammaretrovirus expressing ADA, the SFada/W plasmid DNA was transfected into Phoenix helper free retrovirus producer cells. This is a transient packaging cell line providing the ecotropic env gene separate from the gag and pol genes. The viral supernatant from these cells was then used to stably transduce the murine PG13 cells, derived from the NIH/3T3s. The PG13 cells contain genes for Gag and Pol from murine leukaemia virus (MLV) and in trans the envelope from GaLV (Miller *et al.*, 1991). Thus, the gammaretrovirus generated from these cells are pseudotyped with the GaLV envelope (refer to Figure 1.6).

Flow cytometric analysis demonstrated that nearly 40% of the PG13 cells were transduced (Figure 4.2E). Flow cytometric single cell sorting was performed on the polyclonal PG13 populations to obtain clonal populations of transduced cells. As ADA is expressed intracellularly, this marker could not be used for flow cytometric sorting. Hence, the cells were sorted on the basis of cell viability, as determined by the forward scatter/side scatter characteristics (Figure 4.2A), into 96 well plates. Of the clones that were expanded, 24 were analysed for hADA expression by Western blot and flow cytometry to determine which single cell clones had been transduced. Of the single cell clones analysed, 10 were found to be positive for human ADA expression by both Western blot (Figure 4.2B) and flow cytometry (representative plot in Figure 4.2F), and were therefore considered to be transduced. Flow cytometry confirmed that the cells expanded were probably single cell clones, as they expressed a homogenous amount of ADA. Neither Western Blotting nor intracellular staining for flow cytometry detected murine ADA (data not shown).

The virus produced by the polyclonal PG13 population and the single cell clones was titred to determine which was the better viral producer. To quantify the titres, viral supernatant at different dilutions was used to transduce HeLa cells in one round of transduction, and the level of ADA expression in the HeLa cells was measured three days post-transduction using flow cytometry to determine the proportion of transduced cells (Figure 4.3A-G). The titres of the virus produced from polyclonal

and single cell clone producer cells ranged from approximately $1x10^4$ to $4x10^4$ transducing units/mL as shown in Figure 4.3H. Two of the single cell clones were found not to produce any functional virus although the ADA protein was shown to be expressed as determined by Western blotting (SCCs 6 and 8). The titring on HeLa cells was performed twice to confirm the values. The polyclonal cell population was utilised in some early transduction experiments prior to the generation of single cell clones. As its titre was found to be similar to the best viral producers of the single cell clones, this cell line was continued to be used for some experiments.

As the SFada/W was to be used in a clinical setting to treat ADA deficient patients, virus was produced at a large scale in a good manufacturing practice (GMP) facility in Eufets, Germany. The titre of the clinical grade gammaretrovirus was found to be $8.5x10^4$ transducing units/mL (Figure 4.3H). Several transductions described in this project were performed as indicated using test aliquots of the clinical grade virus.

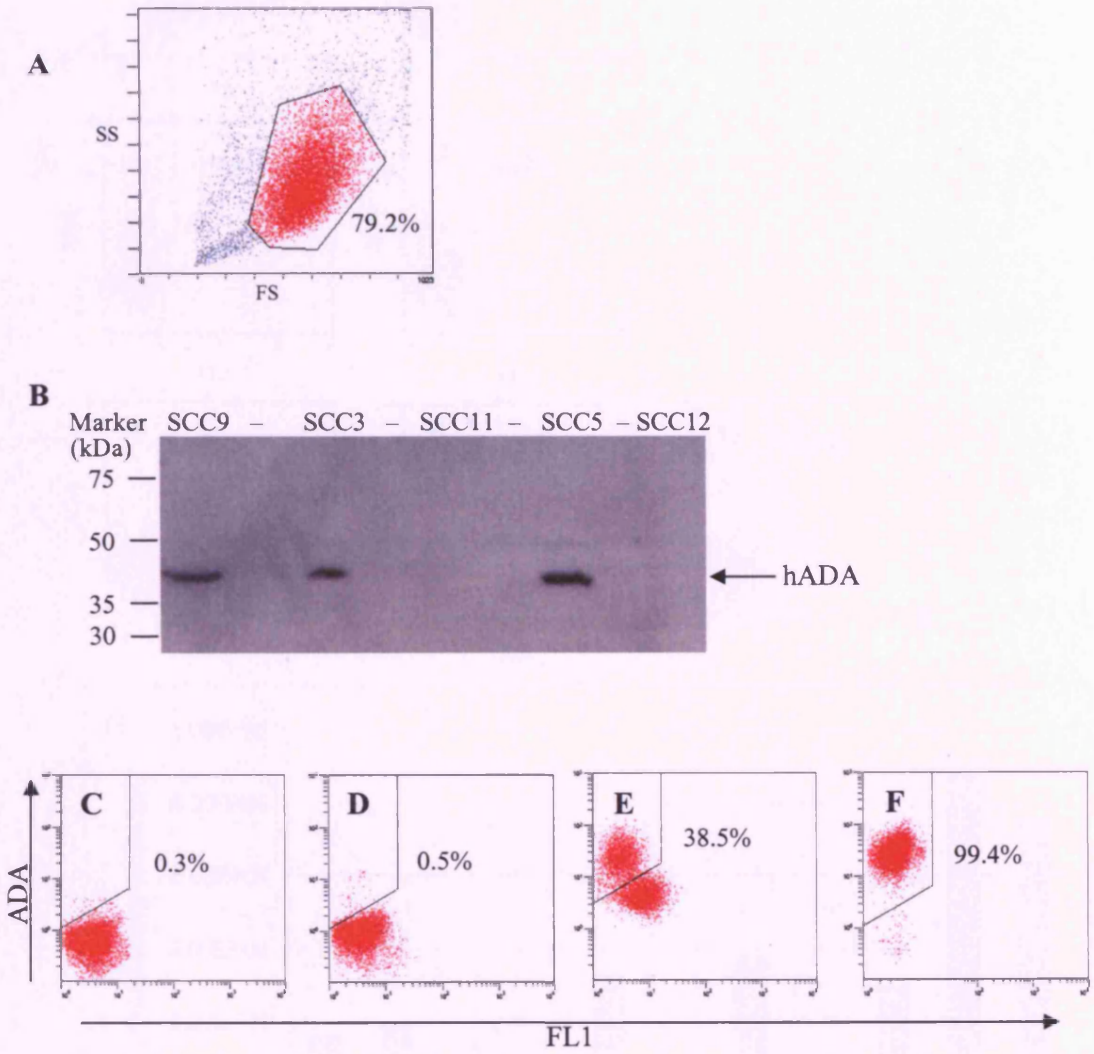


Figure 4.2. Generation of PG13 single cell clones transduced with the ADA gammaretrovirus. PG13 cells were transduced with SFada/W gammaretrovirus. Single cells were sorted into 96-well plates by FACS, expanded and analysed for ADA expression.

A, FACS plot of selected viable PG13 cells. **B,** Representative Western blot of 5 single cell clones. The hADA protein is a monomer of approximately 40kDa. SCC, single cell clone. Representative flow cytometry plots of **C,** secondary antibody only control; **D,** untransduced cells; **E,** the polyclonal population prior to sorting and **F,** single cell clone 5 (**C-F**: The values in the flow cytometry plots refer to the ADA^+ cells).

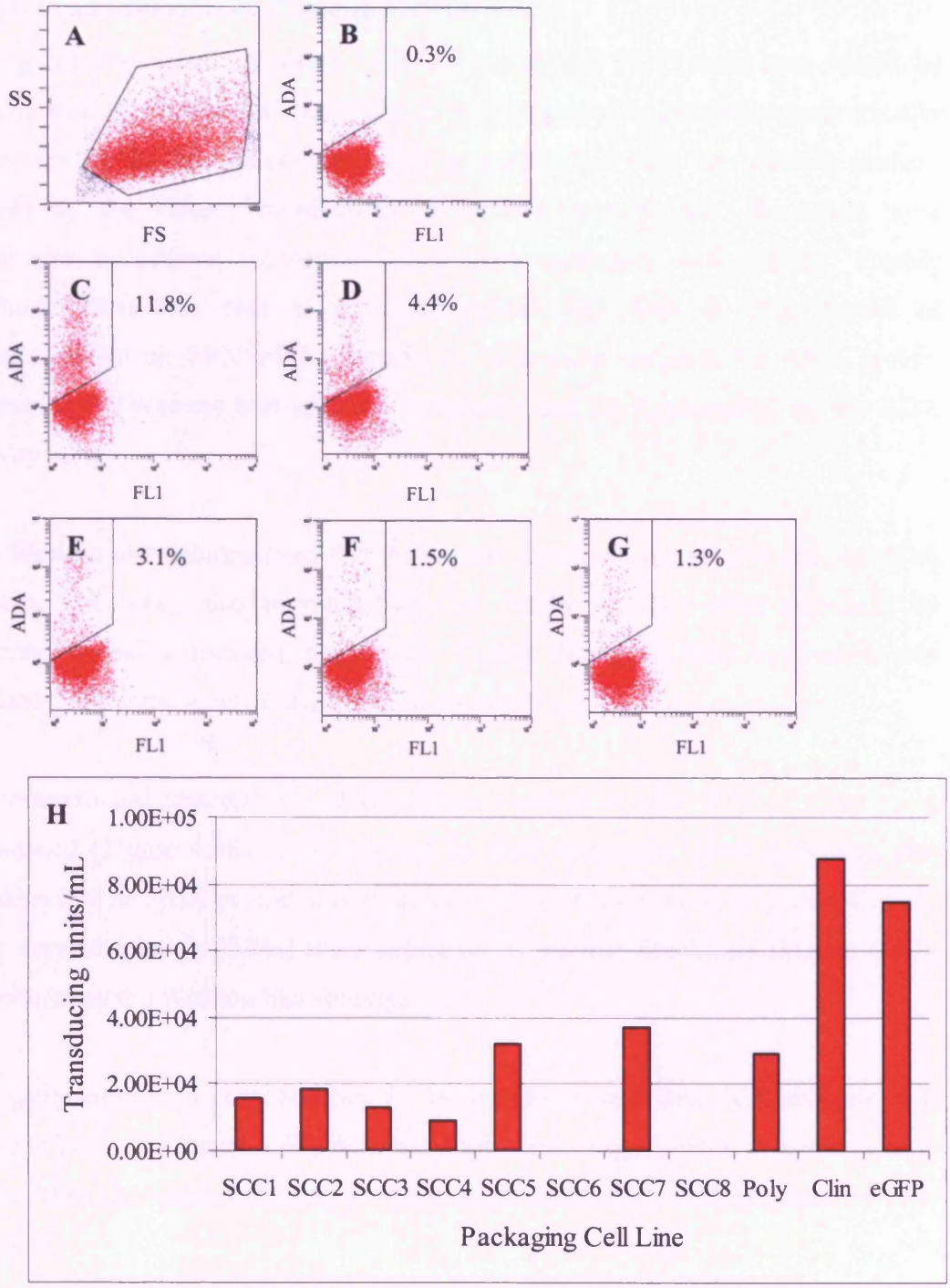


Figure 4.3. Titration of the gammaretrovirus generated by the PG13 packaging cell lines. Each gammaretrovirus produced by the different gammaretroviral producer clones was titrated on HeLa cells to identify the best viral producer.

A, Forward scatter/side scatter plot; **B,** secondary antibody only control. **C-G,** Representative flow cytometry plots of single cell clone 5 titred on HeLa cells. The gammaretroviral supernatant from the PG13 single cell clones was used neat **(C)**, 1:5 diluted **(D)**, 1:25 diluted **(E)**, 1:125 diluted **(F)** and 1:625 diluted **(G)** (**B-G**: The values in the flow cytometry plots refer to the ADA^+ cells). **H,** Titres of gammaretrovirus produced from different gammaretroviral packaging cell lines. The titres were estimated from the transduction efficiency of HeLa cells transduced using serial dilutions of gammaretroviral supernatants. Average values from two separate experiments. Poly, polyclonal PG13 cell population, clin, clinical grade SFada/W gammaretrovirus.

4.2.3 Transduction of Patient Fibroblasts

The gammaretrovirus generated from the polyclonal PG13 cells was tested by transduction of a variety of cell types. It was important to assess the gene transfer efficiency of the gammaretrovirus and to analyse the cells for possible toxicity caused by the virus. Transductions of patient primary skin fibroblasts were performed to achieve reconstitution of ADA expression and activity. Freshly produced virus was used to transduce patient fibroblasts in three rounds of transduction at an MOI of 1. Transduced cells were analysed for ADA protein expression by Western blot and flow cytometry and for functionality by the ADA activity assay.

The Western blot demonstrated that the patient fibroblasts did not express any ADA protein. The assay also revealed that high levels of ADA were expressed by gammaretroviral transduced fibroblasts. Normal fibroblast ADA expression was probably below the level of sensitivity of the test (Figure 4.4A).

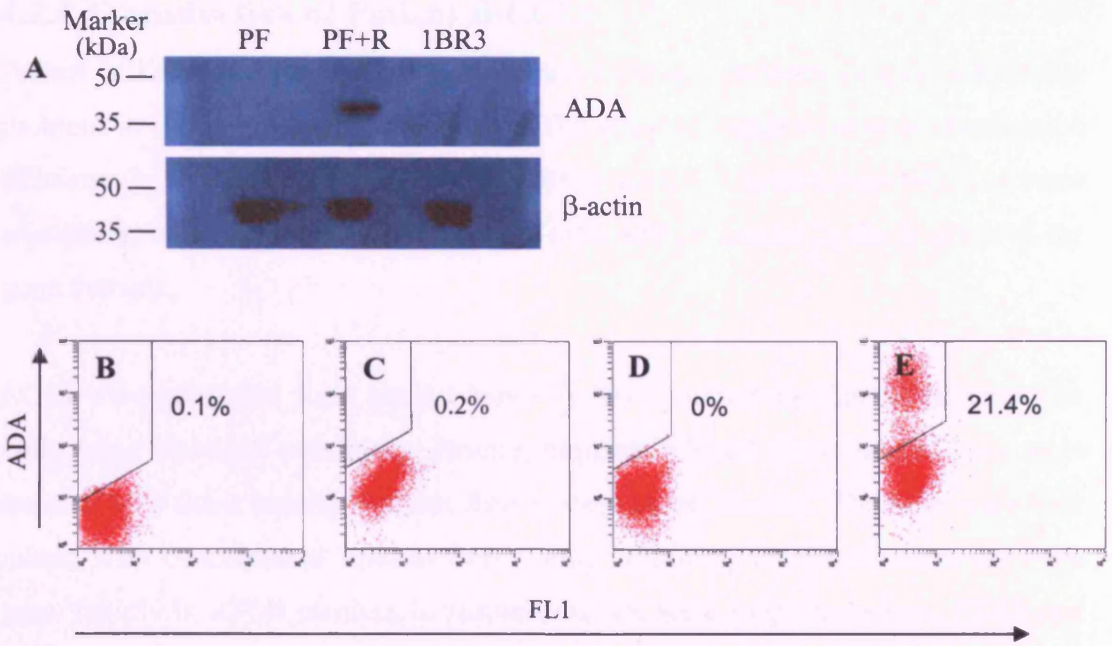
Gammaretroviral transduction of fibroblasts resulted in over 20% of cells being transduced (Figure 4.4E) as determined by flow cytometry. Flow cytometry also revealed that no ADA protein was expressed by patient fibroblasts (Figure 4.4D) and only very low levels (0.2%) were expressed by normal fibroblasts (Figure 4.4C), corroborating the Western blot findings.

The gammaretroviral copy number, as determined by real time PCR analysis, was found to be 0.162 (Figure 4.4F). In other words, on average 1 cell in 6 carried a copy of the ADA transgene, reflecting the percentage transduction as determined by flow cytometry.

The gammaretroviral transduced fibroblasts were found to have an activity of 30,743 nmoles/hr/mg total protein. In comparison, untransduced patient fibroblasts had an activity of 14.3 nmoles/hr/mg total protein and normal fibroblasts 1,756 nmoles/hr/mg total protein (Figure 4.4 F & G). Hence, gammaretroviral transduction resulted in the fibroblasts having an activity 17-fold greater than that of normal. Therefore, although the copy number was quite low, the transductions still resulted in

very high activity levels. The relatively low activity value of the normal fibroblasts suggests that ADA expression in fibroblasts is low and may explain why the ADA protein cannot be detected by Western blot or by flow cytometry analysis. The activity per transgene copy in the transduced patient fibroblasts was found to be 189,772 nmoles/hr/mg total protein.

From these data one can ascertain that the gammaretrovirus is capable of efficiently transducing patient fibroblasts, resulting in high expression of the ADA protein and very high levels of ADA activity.



F

Cells	ADA activity (nmoles/hr/mg protein)	% Transduction	Transgene Copy Number	Activity (nmoles/hr/mg protein) /Copy
1BR3	1,756	N/A	N/A	N/A
P F	14.3	N/A	N/A	N/A
PF+R	30,743	21.4	0.162	189,772

G

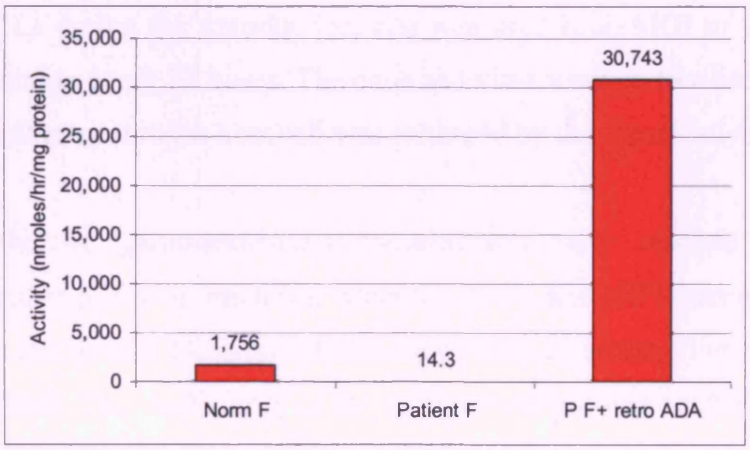


Figure 4.4. Gammaretroviral transduction of patient fibroblasts (PF). The fibroblasts were transduced with gammaretrovirus in three rounds of transduction at an MOI of 1.

A, Western blot of untransduced (PF), gammaretroviral transduced patient fibroblasts (PF+R), and normal fibroblasts (1BR3). The hADA protein has a molecular weight of approximately 40kDa, and β-actin of approximately 42kDa. Flow cytometry plots of **B**, secondary antibody only control; **C**, normal fibroblasts; **D**, untransduced patient fibroblasts; **E**, transduced patient fibroblasts (The values in the flow cytometry plots refer to the ADA^+ cells). **F**, Table depicting the activity of fibroblasts and activity per copy of gammaretroviral transduced fibroblasts. **G**, Chart displaying the activity values of the fibroblasts.

4.2.4 Transduction of Patient B-LCLs

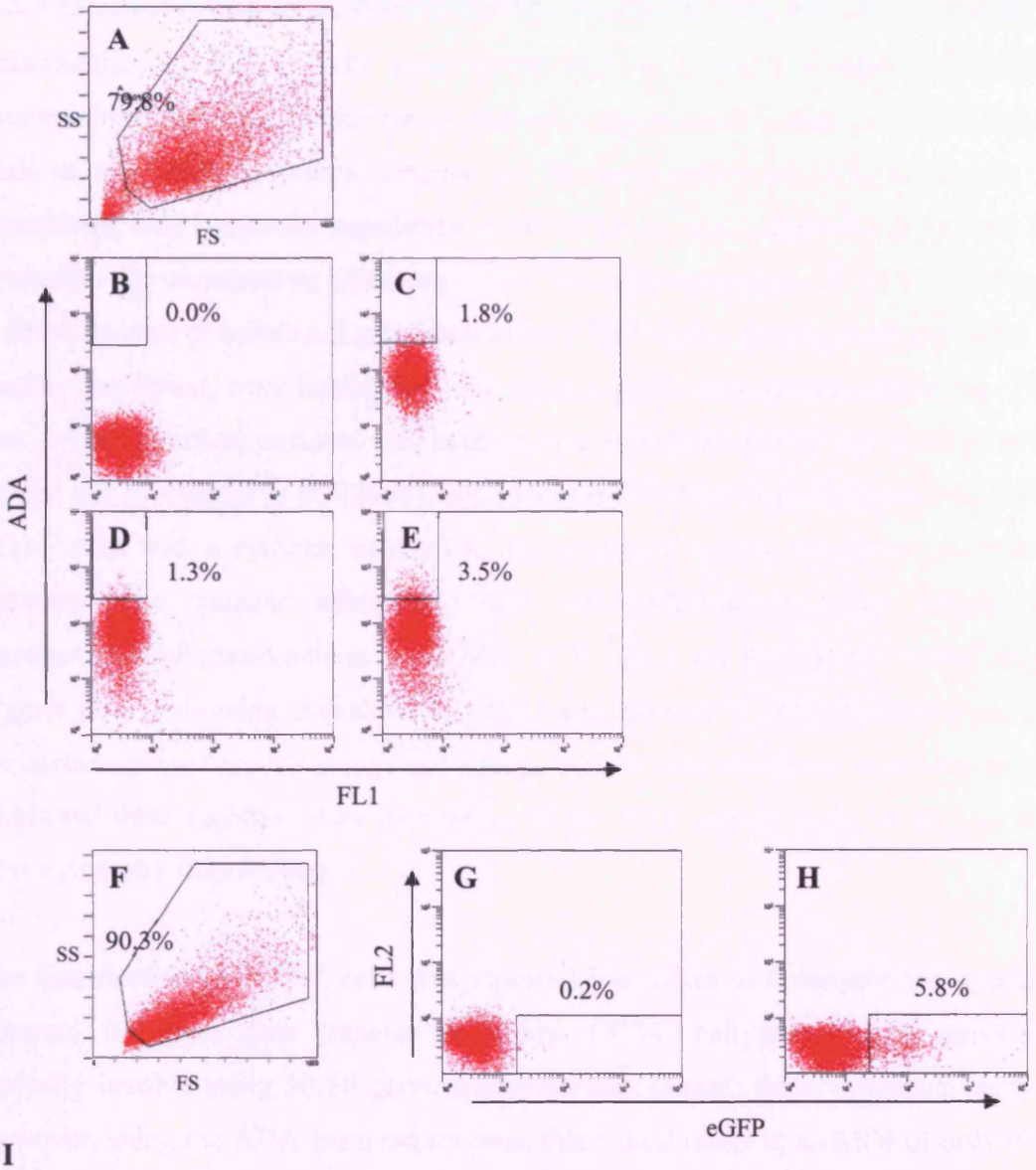
Patient LCLs were transduced with eGFP and ADA-gammaretrovirus using a similar protocol to that used for the fibroblasts. The aim was again to assess transduction efficiency by flow cytometry and ADA activity assays. Transductions of LCLs were assessed as ADA expression in lymphoid cells will be crucial to the success of the gene therapy.

LCLs were generated from patient blood by EBV immortalisation of mononuclear cells using standard techniques. Briefly, peripheral blood mononuclear cells were isolated by a ficoll density gradient from patient blood samples. The cells were then mixed with concentrated Epstein Barr Virus. Following immortalisation, the cells grew rapidly in RPMI medium in suspension, and were found to express CD19 and CD45, but not CD3 as expected. Optimisation of gammaretroviral transduction of the LCLs was attempted by variation of several parameters, including LCL cell density, type of media used for transduction and viral concentration. It was determined that the optimal conditions were to use fibronectin coated plates, and three rounds of transduction with neat gammaretrovirus in X-vivo 10 and the addition of polybrene. The gammaretrovirus was harvested in X-vivo 10 medium to allow for the growth of the LCLs during the transduction, and was used at an MOI of 1 in three rounds of transduction every 12 hours. The cells and virus were co-localised using fibronectin, and viral entry into the host cell was enhanced by the use of polybrene.

Nevertheless, gammaretroviral transductions only resulted in gene transfer efficiencies of 6% or less using either the ADA- and eGFP-gammaretrovirus on both patient and normal LCLs as determined by flow cytometry (Figures 4.5A-H and data not shown). The inefficiency of the gammaretrovirus was confirmed by Western blot analysis, which did not reveal any ADA expression (data not shown). Clinical grade virus generated similar transduction efficiencies. The use of control eGFP gammaretrovirus suggested that the low efficiency of transduction was not due to a problem of the SFada/W virus, but a more general problem in gammaretroviral transduction of B-LCLs.

Real time PCR analysis of transduced LCLs revealed that on average 1 in 10 cells were transduced with the ADA gammaretrovirus (Figure 4.5I), although less than 5% were expressing high levels of ADA as determined by flow cytometry.

The gammaretroviral transduced LCLs were found to have an ADA activity of 3,594 nmoles/hr/mg total protein. This compares with 374 nmoles/hr/mg total protein for patient LCLs and 6,641-8,799 nmoles/hr/mg total protein for normal LCLs (Figure 4.5I). Thus, the gammaretroviral transduced patient LCLs displayed an ADA activity approximately half that of normal. This is consistent with the low transduction efficiency obtained as determined by flow cytometry and real time PCR. The calculated activity value per transgene copy was 37,891 nmoles/hr/mg total protein per copy, five times less than the transduced patient fibroblasts.



I

Cells	ADA activity (nmoles/hr/mg protein)	% Transduction	Transgene Copy Number	Activity (nmoles/hr/mg protein) /Copy
Norm LCLs KG	6,641	N/A	N/A	N/A
Norm LCLs EB	8,799	N/A	N/A	N/A
P3 LCLs	374	N/A	N/A	N/A
P3 LCLs + R	3,594	3.5	0.09475	37,891

Figure 4.5. Gammaretroviral transductions of B-LCLs. The LCLs were transduced at an MOI of 1 in three rounds with clinical ADA or eGFP gammaretrovirus.

A-E, Gammaretroviral-ADA transductions. **A,** Patient 3 B-LCLs forward scatter, side scatter; **B,** secondary antibody only control. ADA expression of **C**, normal LCLs; **D,** untransduced patient 3 B-LCLs; **E,** transduced patient 3 B-LCLs.

F-H, Gammaretroviral-eGFP transductions. **F**, Forward scatter-side scatter plot of patient 3 B-LCLs; **G**, untransduced patient 3 B-LCLs; **H,** transduced patient 3 B-LCLs. (**B-H**: The values in the flow cytometry plots refer to the ADA^+ cells).

I, Activity of B-LCLs and activity per copy of the gammaretroviral (R) transduced patient 3 (P3) B-LCLs.

4.2.5 Optimisation of Protocol for the Transduction of CD34$^+$ Cells

Transductions of CD34$^+$ cells were carried out to analyse the efficiency of the gammaretrovirus on these cells and to optimise the protocol for gene therapy clinical trials to treat ADA deficient patients. As the gammaretrovirus was inefficient at transducing the lymphoid population of B-LCLs, it was especially important to determine the transduction efficiency of the gammaretrovirus on CD34$^+$ cells. Bone marrow derived or mobilised peripheral blood CD34$^+$ cells, derived from patient or a healthy individual, were transduced with clinical grade ADA-gammaretrovirus. The five day transduction protocol was based on the procedure used in the X-SCID trial carried out by Gaspar *et al.* (2004), and included two days of pre-stimulation of the CD34$^+$ cells with a cytokine cocktail to encourage the cells to divide and therefore improve gene transfer efficiency. This was followed by three rounds of gammaretroviral transductions at an MOI of 0.5 or 1 on Retronectin coated bags (Figure 4.6). Following transduction, cells were cultured in methylcellulose media for haematopoietic colony assays and a proportion of cells were kept in culture for an additional three days to allow expression of the ADA protein prior to analysis by flow cytometry (day 8 data).

The transduction of CD34$^+$ cells was repeated four times to determine the optimal protocol for good gene transfer efficiency. CD34$^+$ cell transduction protocols typically involve using 50:50 gammaretroviral supernatant: fresh activation media. However, using the ADA gammaretrovirus, this would result in an MOI of only 0.5. Hence, experiments were performed using 90:10 gammaretroviral supernatant: fresh activation media (MOI 1) to determine whether cell viability could be maintained using this high volume of viral supernatant. Flow cytometry analysis demonstrated that using the higher MOI resulted in improved transduction efficiency (Figure 4.7A), which increased from 29.3% to 36.7% as analysed on day 8. Trypan blue analysis also showed that cell viability remained high at around 90% (data not shown).

Similar numbers of the different types of methylcellulose colonies were observed for both untransduced and transduced CD34$^+$ cells (Figure 4.7B). Thus, the phenotypes of the progenitor cells were not altered by viral transduction and their differentiation

capacity remained the same after transduction. These data also importantly demonstrate that there is no toxicity related to the transduction. PCR on the methylcellulose colonies revealed that approximately 50% of the colonies were transduced (Figure 4.7C), slightly higher than the transduction efficiency estimated by flow cytometry. No preferential transduction into a particular lineage was detected by the PCR, although this should be confirmed by repeating this experiment which was only performed once.

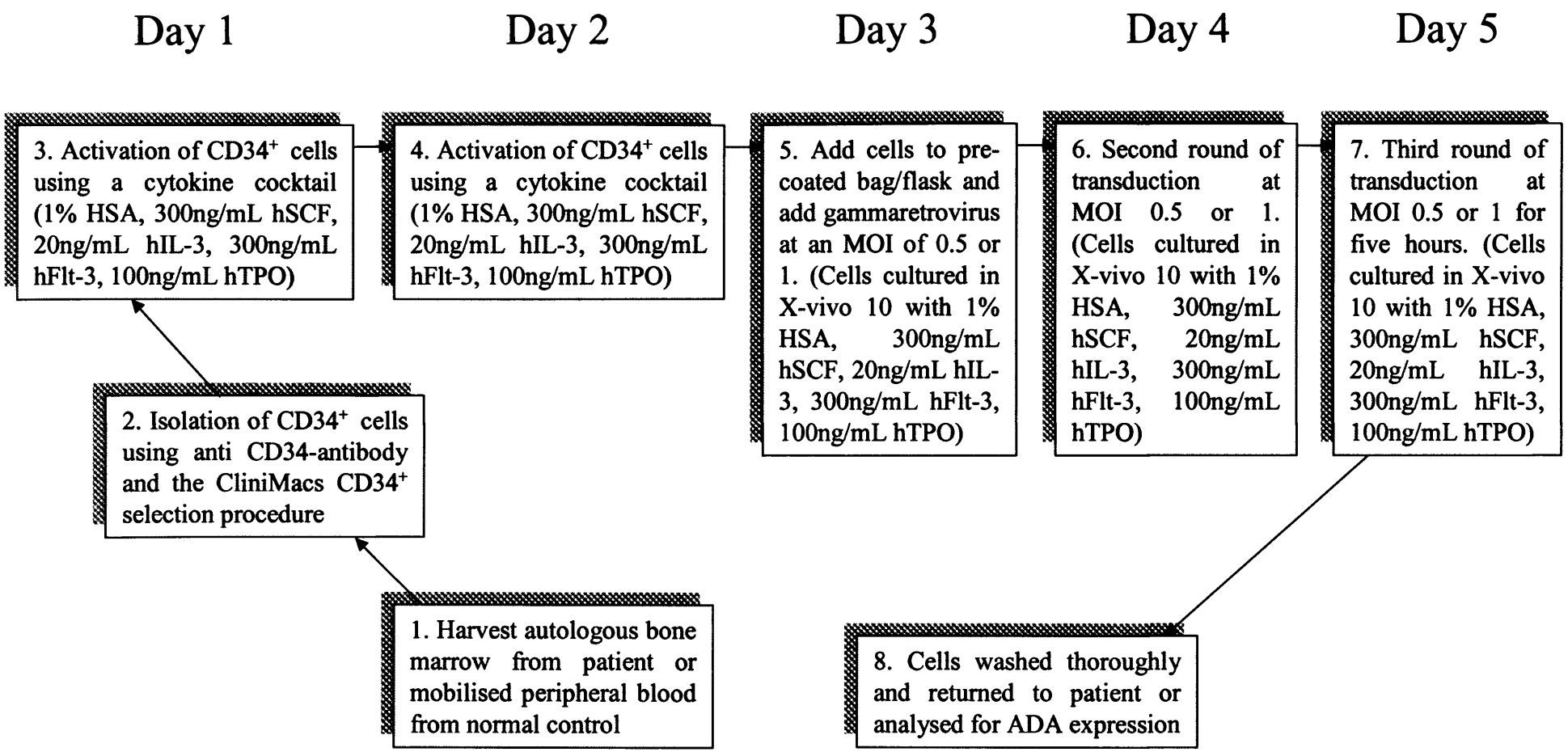


Figure 4.6. Protocol for transduction of CD34+ cells using a gammaretroviral vector. Based on X-SCID clinical trial reported by Gaspar *et al.*, 2004.

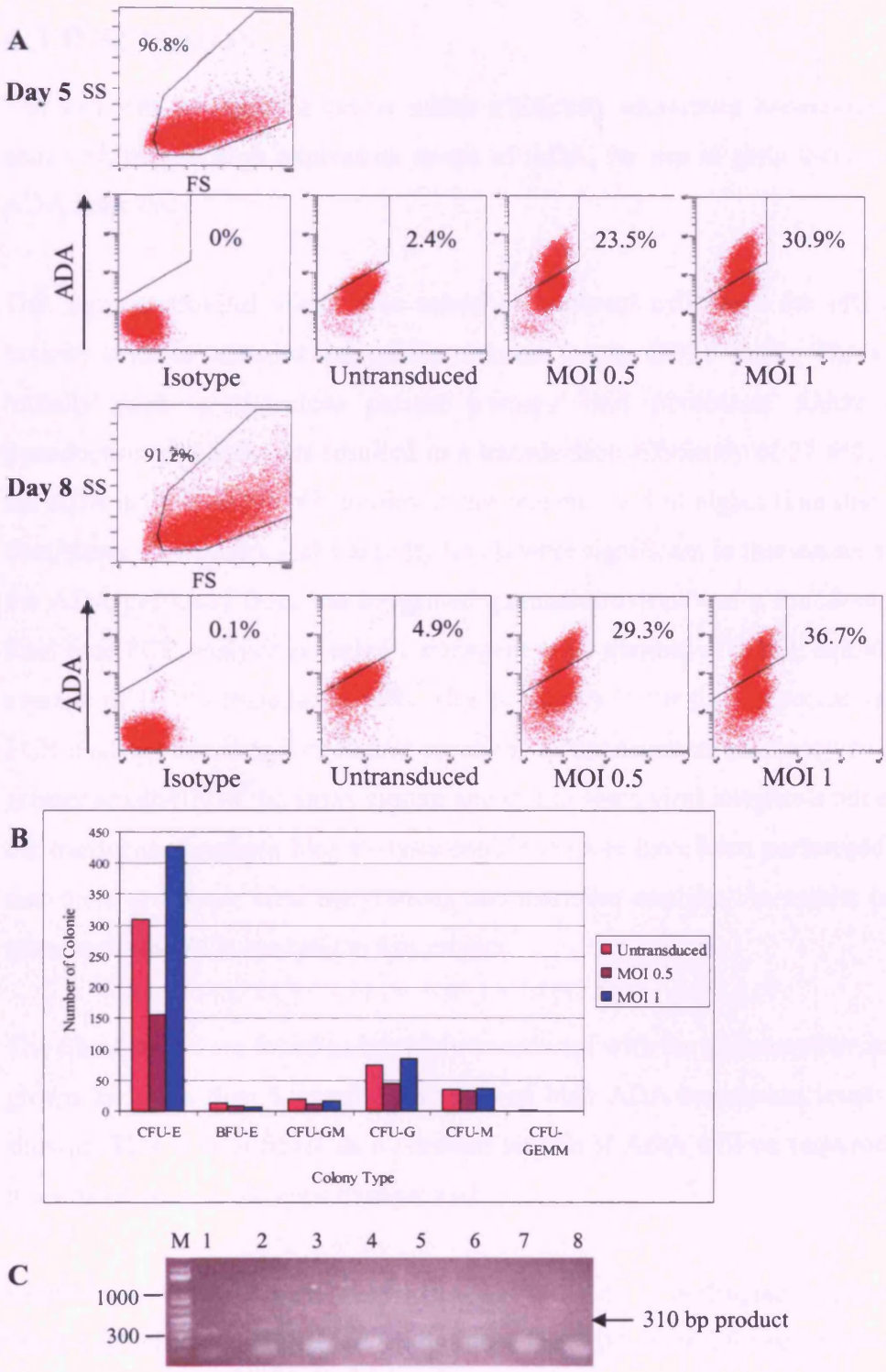


Figure 4.7. Gammaretroviral transduction of normal CD34⁺ cells. Activated peripheral blood derived CD34⁺ cells were transduced with clinical grade ADA gammaretrovirus at an MOI of 0.5 or 1 following the protocol depicted in the previous figure. **A,** Gammaretroviral transduction efficiency of normal CD34⁺ cells at two different MOIs. Day 5 is following two rounds of transduction and day 8 is following 3 rounds of transduction and allowing time for expression of the transgene. (The values in the bottom 4 flow cytometry plots of days 5 and 8 refer to the ADA⁺ cells). **B,** Numbers of different haematopoietic colony types grown from 1,000 untransduced or transduced CD34⁺ cells (at MOI 0.5 or 1) in methylcellulose media. **C,** Representative PCR of methylcellulose colonies. M, 1kb ladder. The colonies that were found to be positive were 1, 2, 5 and 6. Product size is 310 base pairs.

4.3 DISCUSSION

The aim was to design a vector which efficiently transduces haematopoietic stem cells resulting in high expression levels of ADA, for use in gene therapy trials for ADA deficiency.

The gammaretroviral vector was tested on different cell types for efficiency and toxicity prior to transduction of the ultimate target, $CD34^+$ cells. The vector was initially used to transduce patient primary skin fibroblasts. Gammaretroviral transduction of fibroblasts resulted in a transduction efficiency of 21.4%, increasing the ADA activity to 30,743 nmoles/hr/mg protein, 17-fold higher than that of normal fibroblasts. These high ADA activity levels were significant as this demonstrated that the ADA produced from the integrated gammaretrovirus was a functional enzyme. Real time PCR analysis revealed a transgene copy number of 0.162, equivalent to an average of 16.2% transduced cells. This is slightly lower than expected as real time PCR analysis usually gives higher estimates of transduction efficiency as a result of greater sensitivity of the assay system and due to some viral integrants not expressing the transgene. Southern blot analysis could therefore have been performed to ensure that there are intact viral integrations and therefore confirm the results of this and other real time PCR analysis in this project.

The fibroblasts were found to be stably transduced with the gammaretrovirus, as cells grown for more than 6 months still showed high ADA expression levels (data not shown). This is significant as a constant source of ADA will be required from the transduced cells in the gene therapy trial.

Gammaretroviral transduction of LCLs was found to be inefficient as a maximum of 6% transduction was achieved using either the ADA or eGFP vector. However, the gammaretroviral transduced LCLs were found to have an ADA activity of 3,594 nmoles/hr/mg total protein, approximately half that of normal LCLs. It is not clear as to why greater transduction efficiencies were not achieved as the cells were rapidly dividing and would be expected to be susceptible to gammaretroviral transduction, but it is possible that the LCLs do not express high levels of the GaLV receptor (Pit-1) on the cell surface for the virus to bind to. The efficiency of gene transfer seen in

our experiments cannot be compared with several published values as many groups tend to use vectors expressing the Neomycin resistance gene and expand the cells in G418 selection media following transductions (Thrasher *et al.*, 1992; Taylor *et al.*, 1996; Akatsuka *et al.*, 2002).

The gammaretroviral vector used in our study was therefore found to efficiently transduce patient fibroblasts resulting in high levels of ADA expression and activity, but was less efficient at transducing patient haematopoietic B-LCLs. Hence, the viral vector was tested extensively on normal and patient $CD34^+$ cells prior to initiation of gene therapy trials. The cells were pre-activated using a cytokine cocktail to induce cell division necessary for gammaretroviral transduction. In each experiment, the gammaretrovirus was found to be efficient at transducing the haematopoietic cells. It was found that using an MOI of 1, which involved using 90% viral supernatant, resulted in a transduction efficiency of 36.7% and good cell viability. It was therefore decided to use this MOI for the gene therapy trial. PCR of the methylcellulose colonies revealed transgene expressing cells in all haematopoietic subsets, importantly suggesting that the gammaretrovirus does not show preferential integration into a particular cell type. Approximately 50% of the colonies were found to be transduced by this method, a transduction efficiency estimate slightly higher than the flow cytometry results. Therefore, it is likely that the transduction efficiency with the gammaretrovirus is slightly higher than the 36.7% observed at an MOI of 1 by flow cytometry.

Although slightly different transduction efficiencies have been calculated by the different analysis methods, it has been demonstrated that the HSCs have been efficiently transduced. The transduction efficiency using gammaretrovirus at an MOI of 1 is in the region of 35-50%. These values are in line with published figures of $CD34^+$ transduction which are also in the region of 30-50% (Schilz *et al.*, 2000; Pollok *et al.*, 2001). Interestingly, the successful gene therapy trial for ADA deficiency by Aiuti *et al.*, 2002a, reported that 25 and 21% transduced cells were reinfused into the two patients. Hence the transduction efficiencies achieved in our trial runs should be sufficient for therapeutic effects in ADA deficient patients.

In conclusion, an ADA gammaretroviral vector has been generated which adequately transduces different cell types without causing toxicity. The clinical gammaretroviral vector was utilised to optimise the protocol for transduction of $CD34^+$ cells, and was found to transduce these cells with good transduction efficiency and without cellular toxicity caused by gammaretroviral mediated expression of the ADA transgene. This gammaretroviral vector will therefore be used in clinical gene therapy trials for ADA deficiency. Hence, based on the initial results obtained for the ADA-gammaretrovirus, it is expected that this vector will not result in cell toxicity but will be efficient at transducing the $CD34^+$ cells and generate therapeutic levels of ADA.

5

ANALYSIS OF GENE THERAPY TREATMENT FOR ADA DEFICIENCY

5.1 INTRODUCTION

Patient 3 was enrolled in the phase I gene therapy trial, as in spite of the PEG-ADA treatment, his lymphocyte counts were low and his naïve population of cells was diminishing. The gene therapy protocol was approved by the UK Gene Therapy Advisory Committee, the Medicines Control Agency (now Medicine and Healthcare products Regulatory Agency) and by the local Institutional Research Ethics Committee. Entry criteria were confirmation of diagnosis of ADA deficiency by mutation analysis or increased levels of dATP, absence of a matched related or unrelated donor, and lack of response to PEG-ADA treatment. Prior to initiation of gene therapy, written informed consent was obtained from parents following discussion of alternative treatment options.

A number of gene therapy trials for ADA-SCID have been performed. These studies have involved the *ex vivo* transduction of target cells, which were either autologous T cells, bone marrow derived $CD34^+$ cells or umbilical cord blood $CD34^+$ cells, with a gammaretroviral vector encoding the ADA transgene. If $CD34^+$ stem cells have been successfully targeted, these should be capable of reconstituting the immune system and in so doing will allow haematopoietic detoxification. This would encourage production of new immune cells to protect the patient from infections without the need for further medication. As the gammaretrovirus integrates the ADA transgene into the genome of the host cell, the expression of ADA should persist long term. This would result in a life-long treatment for ADA deficient patients and therefore obviate the need for further medications or gene therapy procedures.

Aiuti *et al.*, (2002b) demonstrated that the gradual withdrawal of PEG-ADA in a patient who did not show immune recovery prior to or following gene therapy, can result in improved immune function. Following discontinuation of PEG-ADA, the proportion of transduced T-lymphocytes and the absolute T cell counts increased. Along with an increase in PBL ADA activity, this allowed for the complete restoration of T cell functions. Eventually, metabolite correction was observed in erythrocytes, suggesting that a critical number of transduced lymphocytes may be required for systemic detoxification. Following these results, a successful gene therapy trial for ADA deficiency was performed by Aiuti *et al.* (2002a). Their

protocol was carried out as described above, however, there were some significant differences from previous gene therapy trials for ADA-SCID. Firstly, the patients did not receive PEG-ADA prior to or following gene therapy, thereby creating an environment where the transduced cells have a selective advantage. The patients also received a low dose of conditioning in order to make more space for the transduced cells. The two patients treated with this protocol are currently doing well, showing immune reconstitution. Following the successful treatment of these two patients, 3 further patients have been treated with similarly encouraging results (Aiuti *et al.*, 2005).

Hence, after three years of PEG-ADA therapy patient 3 was treated with gene therapy using the protocol developed in the previous chapter. The gene therapy procedure mimics that of Aiuti *et al.*, as patients treated using this protocol have shown good immune recovery. We analysed in detail the immune recovery following gene therapy in patient 3. This patient was the first in which PEG-ADA was electively stopped prior to gene therapy, whereas in the study by Aiuti *et al.* the patients did not commence PEG-ADA. A number of assays were performed to assess the progress of patient 3: measurements of lymphocyte counts (performed by the Clinical Laboratory at Great Ormond Street Hospital) and dATP concentrations (performed by Lynette Fairbanks at the Purine Research Laboratory, Guys' Hospital), immunophenotype panels by flow cytometry to analyse the production of naïve immune cells, spectratyping (performed with the help of Stuart Adams, ICH) and TREC analyses (performed with the help of Doug King, ICH). The five-day gene therapy procedure was also assessed by flow cytometry and real-time PCR analysis to verify that an adequate number of cells were transduced.

5.2 RESULTS

5.2.1 Gene Therapy Procedure for Patient 3

In order to maximise the selective advantage of transduced cells, PEG-ADA therapy was discontinued one month prior to gene therapy. Bone marrow was harvested on day -4 and the patient was then conditioned with one dose of melphalan on day -2 ($140mg/m^2$) to allow for maximum engraftment of the transduced cells (Appendix 2).

$CD34^+$ cells were isolated from patient bone marrow, activated for two days to induce cell division and exposed to the SFada/W virus in three rounds of transduction, and $13x10^6$ autologous cells/kg ($247x10^6$ cells in total) were then returned to the patient on day 5 of the procedure (refer to Figure 4.6). Throughout the transduction procedure, samples of cells were analysed by flow cytometry for ADA and CD34 expression. An aliquot of cells was also kept in culture for a further 3 days after the end of the transduction procedure, to allow time for expression of the transgene for analysis.

On day one of the transduction procedure a large proportion of the cells, 78.3%, were CD34 positive. This proportion of cells decreased during the procedure as the cells were continually activated, causing them to differentiate. On day 5, the day of the reinfusion of transduced cells, the proportion of $CD34^+$ cells had decreased to 37.1%. A small aliquot of cells maintained in culture after the infusion of the transduced cells revealed that the percentage of ADA positive cells was 25.2%. Therefore, 25.2% ($3.3x10^6$ cells/kg) of the infused cells were transduced and the percentage of $CD34^+$ cells upon reinfusion was 37.1% ($4.8x10^6$ cells/kg) (Figure 5.1). However, one cannot estimate the percentage of transduced $CD34^+$ cells on the day the cells are returned to the patient, as the cells would not have had enough time to express the transgene. Hence, ADA expression is analysed on day 8, however by that time the CD34 expression has significantly decreased from the time the cells were reinfused. Thus, the percentage transduced $CD34^+$ cells is difficult to estimate, as it is clear that the maintenance of $CD34^+$ cells in activation media for an extended period of time decreases the expression of CD34.

Methylcellulose cultures were set up using the aliquot of cells that was kept in culture, and the numbers of the different colonies obtained from 1,000 plated cells are displayed in Figure 5.2A. The proportions of the different types of colonies are almost identical to both the untransduced and transduced cells in the previous gammaretroviral-CD34 experiment shown in Figure 4.7B. Therefore, importantly the phenotype of the progenitor cells does not appear to have been altered by the transduction procedure.

PCR was performed on the 20 colonies that were picked (Figure 5.2B). Of these colonies, 7 were found to be positive resulting in a calculated transduction efficiency of 35%. This corroborated the transduction efficiency estimate obtained by flow cytometry, considering that PCR analysis would be expected to give a slightly higher estimate as some integrated viruses do not result in expression of the transgene. Therefore, the transduction efficiency was in the region of 25-35%. The colonies found to be positive by PCR were further analysed by real time PCR analysis, using β-actin as the control. This revealed that the average copy number of the transduced cells was 2.33 and that the copy number ranged from 1 to 4 (Figure 5.2C).

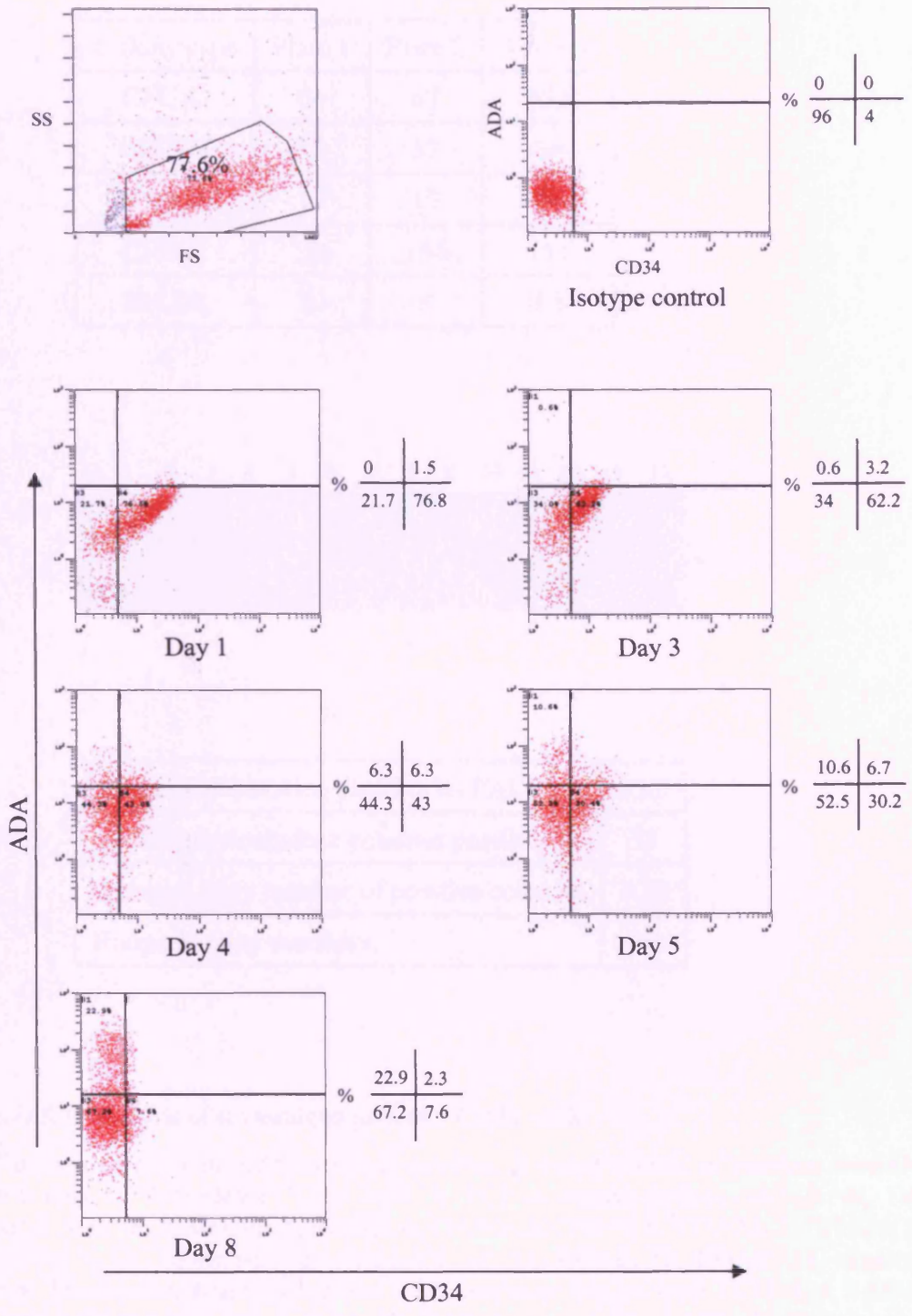


Figure 5.1. Expression of ADA and CD34 of patient 3 haematopoietic cells during the gene therapy transduction procedure. Cells were maintained in activation media for two days prior to gammaretroviral transduction. The cells were infused into the patient on day 5. Following the 5 day transduction procedure a small aliquot of cells were kept in culture for a further 3 days to allow expression of the transgene for analysis (day 8 results).

A

Colony type	Plate 1	Plate 2	Average
CFU-G	66	67	66.5
CFU-M	35	37	36
CFU-GM	17	19	18
CFU-E	136	166	151
BFU-E	10	9	9.5

B

C

% of cell population positive by FACS	25.2
% of methylcellulose colonies positive	35
Average copy number of positive colonies	2.33
Range of copy numbers	1-4

Figure 5.2. Analysis of transduced patient 3 CD34⁺ cells.

A, Numbers of different methylcellulose colonies. Average number of colonies observed from a total of 1000 plated CD34⁺ cells. **B,** PCR of methylcellulose colonies. M, 1kb ladder, 1, CFU-E; 2, BFU-E; 3, BFU-E; 4, CFU-G; 5, CFU-G; 6, CFU-M; 7, CFU-M; 8, CFU-GM; 9, CFU-GM; 10, CFU-E; 11, CFU-E; 12, BFU-E; 13, BFU-E; 14, negative control. The colonies which were found to be positive on this gel were 2 CFU-E's, 3 BFU-E's, and 1 CFU-G (nos. 1, 2, 3, 4, 11, 12). Product size is 310 base pairs. **C,** Transduction efficiency of patient 3 CD34⁺ cells. Transduction efficiency was estimated by flow cytometry and PCR of methylcellulose colonies. Copy numbers were estimated by real-time quantitative PCR analysis.

5.2.2 Analysis of Transgene Presence in Lymphocytes

Following gene therapy, it was important to determine whether the transgene was present in the haematopoietic lineages and whether it persisted. Patient blood samples regularly obtained following gene therapy were sorted by FACS into different cell lineages. Each cell type of interest was then analysed for the presence of the ADA transgene using real time PCR analysis, and an estimated copy number was calculated (Figure 5.3A). A graph was plotted to display the estimated copy number in each cell type over time (Figure 5.3B). The ADA transgene was detected in all cell types analysed, which were T cells, B cells, NK cells and granulocytes up to 16 months post gene therapy. The highest transgene copy number was found to be in the T cells and NK cells, which at 16 months post gene therapy was 0.528 and 0.394 respectively. No analysis was done for B cells during 6 months when these cells could not be detected as a result of a treatment for an EBV infection as discussed in section 5.3.

A

Cell Types	1 month	2 months	4 months	6 months	9 months	11 months	13 months	16 months
CD3	0.009	0.014	0.429	0.319	0.212	0.095	0.437	0.528
CD19	0.051	0.076	ND	ND	0.038	0.047	0.019	0.077
NK	ND	0.131	0.663	0.587	0.210	ND	ND	0.394
Gran	0.025	0.015	0.002	0.002	0.009	0.001	0.001	ND

B

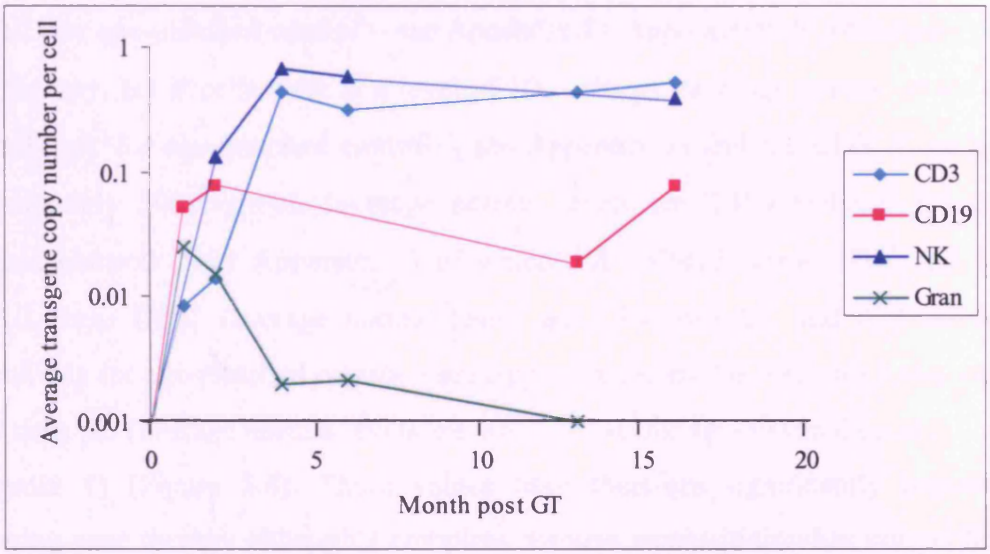


Figure 5.3. Average copy number of ADA transgene in patient 3 haematopoietic cells.

A, Copy numbers for the different cell types in the months following gene therapy as determined by real time PCR (each value is an average of triplicate readings). ND, not determined. **B,** Copy numbers over time (Gran, granulocytes).

5.2.3 Analysis of Lymphocyte Counts and Metabolite Levels

Patient 3 was not responding well to PEG-ADA therapy, evident from his low lymphocyte counts. Following gene therapy, patient 3 had an EBV infection, resulting in a substantial increase in his ALC and in particular his $CD8^+$ T cells, an indicator of viral infection. Analysis of the $CD8^+$ T cells demonstrated expansion of a single clone (data not shown). Once the infection had cleared, his absolute lymphocyte count increased and has now reached a level of approximately 800 cells/μL, slightly higher than prior to gene therapy (average normal levels are 3,300 cells/μL for age-matched control – see Appendix 1). Approximately 14 months post gene therapy, his B cells were at a level of 100 cells/μL (average normal levels are 800 cells/μL for age-matched control – see Appendix 1) and his $CD3^+$ T cells at approximately 500 cells/μL (average normal levels are 2,300 cells/μL for age-matched control – see Appendix 1) of which 150 cells/μL were $CD4^+$ and 350 cells/μL were $CD8^+$ (average normal levels are 1,300 cells/μL and 800 cells/μL respectively for age-matched control – see Appendix 1), and his NK cells had a value of 40 cells/μL (average normal levels are 400 cells/μL for age-matched control – see Appendix 1) (Figure 5.4). These values have therefore significantly improved following gene therapy although a complete immune reconstitution has not yet been observed.

Prior to PEG-ADA therapy, patient 3 showed very high toxic levels of the metabolite dATP. However, the PEG-ADA treatment resulted in efficient removal of dATP, and levels which had been at 1,500 μmol/L were rapidly reduced to nearly undetectable levels within a few months. Throughout the PEG-ADA treatment, dATP was kept at a very low level. As the PEG-ADA was withdrawn prior to gene therapy, these levels increased slightly to approximately 100 μmol/L. However, this dATP level was not a true reading as the patient had been given a blood transfusion prior to this measurement, lowering the metabolite levels. The level of dATP would therefore usually be higher as a result of the withdrawal of PEG-ADA. Following gene therapy, the levels of dATP were slightly higher than during the PEG-ADA treatment. However, the dATP has been maintained at levels similar to those observed following bone marrow transplantations (Figure 5.5).

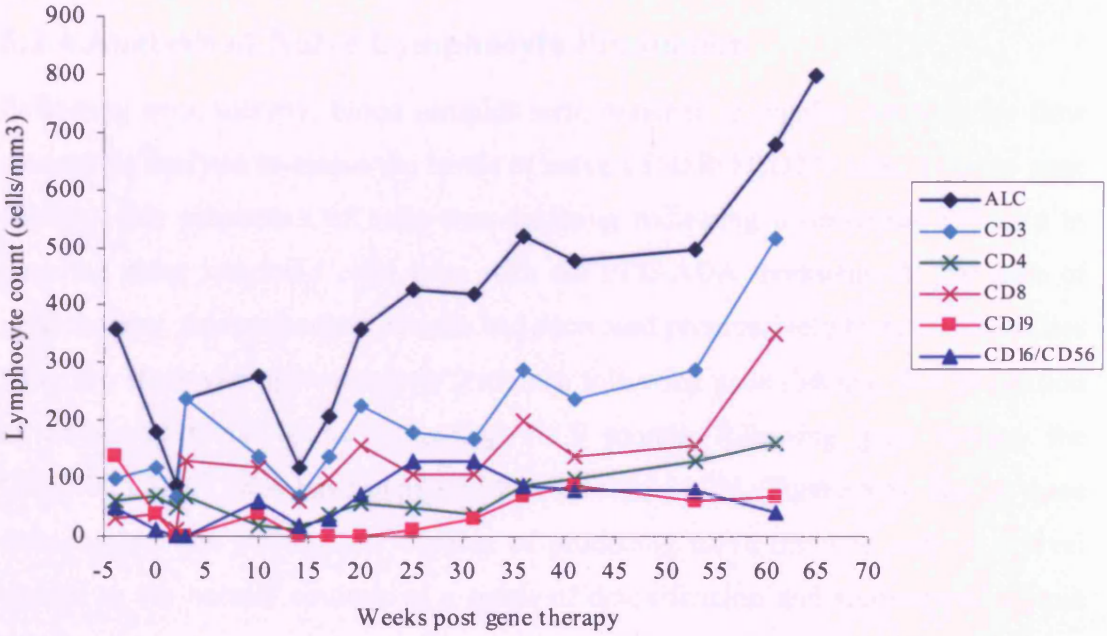


Figure 5.4. Lymphocyte counts for patient 3 following gene therapy. Blood samples were taken at regular intervals and analysed by flow cytometry to estimate the number of cells per μL of the lymphocyte subsets. The period of EBV infection and subsequent increased numbers of T cells were omitted from this graph to improve the clarity of the figure to demonstrate the trends of the lymphocyte counts.

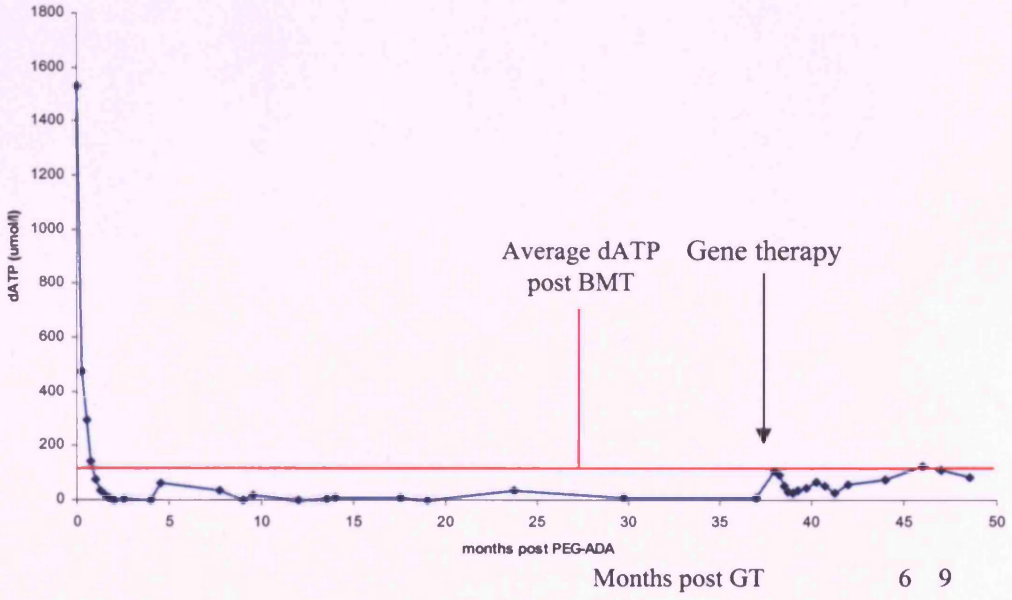


Figure 5.5. Patient 3 dATP levels (μmol/L) following PEG-ADA and gene therapy. During PEG-ADA treatment, the dATP levels decreased to almost undetectable levels. Following gene therapy the patient has low levels of dATP, similar to what is seen post bone marrow transplantations.

5.2.4 Analysis of Naïve Lymphocyte Production

Following gene therapy, blood samples were obtained at regular intervals for flow cytometric analysis to assess the levels of naïve $CD45RO^{-}CD27^{+}$ cells. Prior to gene therapy, this proportion of cells was declining indicating a decreased capacity to generate naïve lymphoid cells even with the PEG-ADA treatment. At the time of gene therapy, this proportion of cells had decreased progressively from 17.5% to less than 5%. However, approximately 2 months following gene therapy, this proportion of cells was found to be increasing. At 9 months following gene therapy the $CD45RO^{-}CD27^{+}$ cells have increased to a level of 31.2% (Figure 5.6). Hence these data suggest that patient 3 is capable of producing naïve immune cells at a level similar to the normal control, as a result of detoxification and recovery of thymic function.

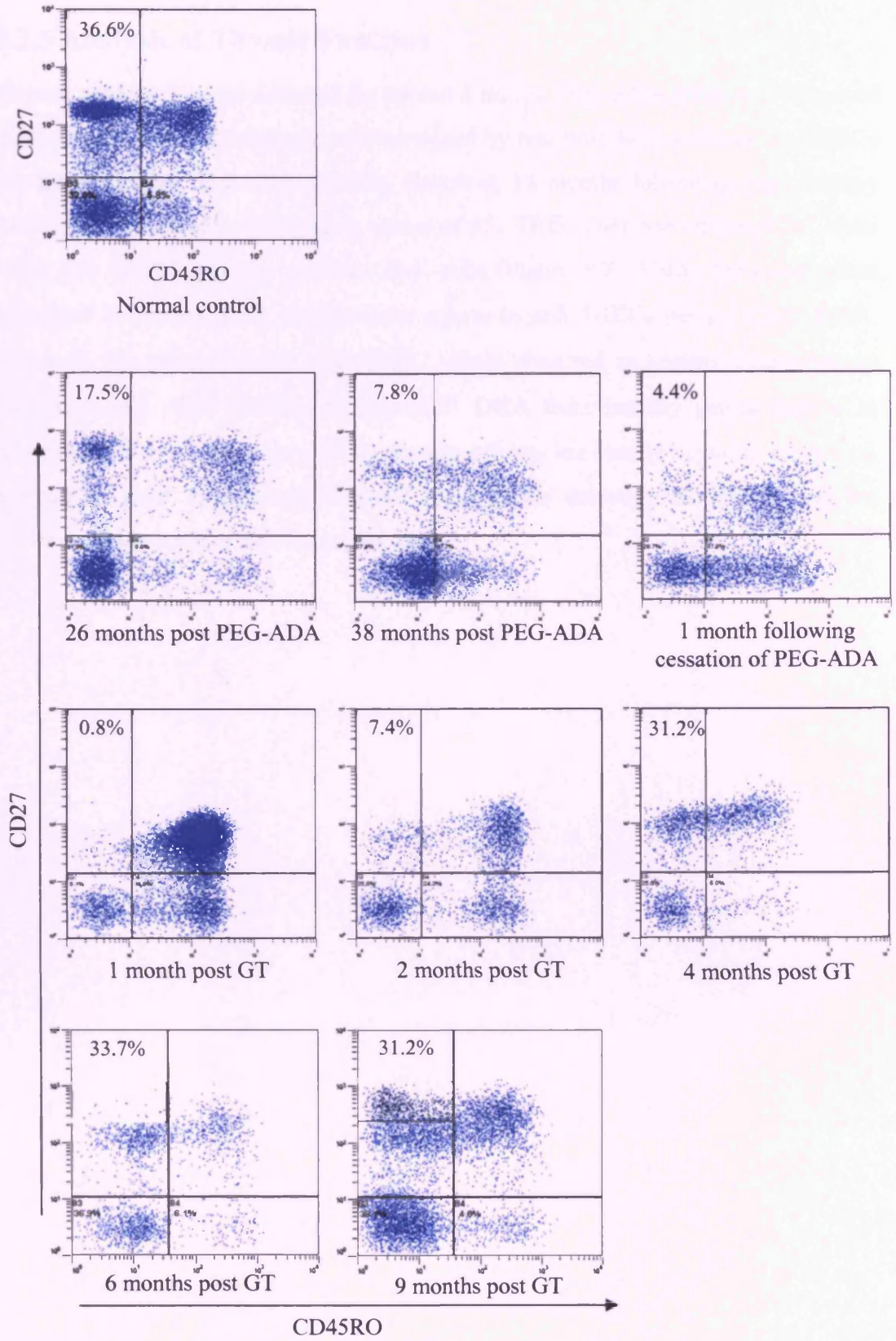


Figure 5.6. Determination of the proportion of patient 3 naïve lymphocytes during PEG-ADA (second row of plots) and following gene therapy (third and fourth rows of plots). The $CD45RO^{-}CD27^{+}$ population of cells represents naïve lymphocytes being produced. PEG-ADA is discontinued at 38 months in preparation for gene therapy. The gene therapy procedure is carried out 1 month following the discontinuation of PEG-ADA.

5.2.5 Analysis of Thymic Function

Thymic activity was not detected for patient 3 during PEG-ADA treatment or indeed for 9 months post gene therapy, as determined by real time PCR analysis for TRECs in separated $CD4^+$ and $CD8^+$ T cells. However, 14 months following gene therapy TREC activity could be detected at values of 951 TRECs per one million $CD8^+$ cells and 1,859 TRECs per one million $CD4^+$ cells (Figure 5.7). Using the calculations described in section 3.2.3, these values equate to 468 TRECs per μg $CD3^+$ DNA. Although this value is lower than TREC values observed in healthy young donors (approximately 4000 TRECs per μg $CD3^+$ DNA from healthy young donors as reported by Talvensaari *et al.*, 2002), thymic activity has clearly begun to recover as a result of gene therapy, as indicated by both the detection of TRECs and the increased proportion of naïve lymphocytes.

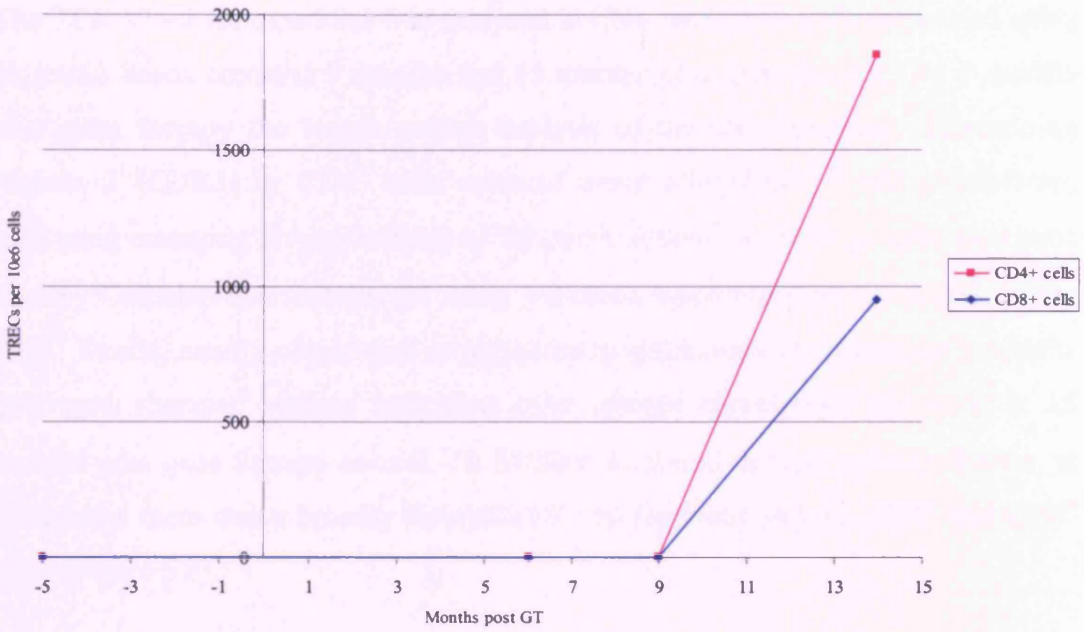
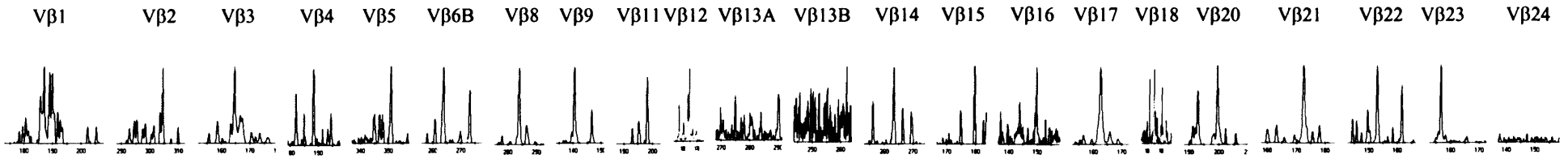


Figure 5.7. TREC values for patient 3 pre and post gene therapy. Patient blood samples were collected at regular intervals and $CD4^+$ and $CD8^+$ cells were separated using magnetic beads column. Real-time PCR analysis was performed on the genomic DNA to estimate the numbers of TRECs in the samples.

5.2.6 Analysis of TCR Diversity

The TCR Vβ-chain repertoire was analysed in $CD4^+$ and $CD8^+$ cells (separated using magnetic beads column) 5 months and 15 months post gene therapy. At 5 months post gene therapy the length pattern analysis of the complementary determining regions 3 (CDR3) in $CD4^+$ cells revealed some oligoclonal T cell populations, indicating emerging diversifications of Vβ-chain repertoires. At 15 months post gene therapy Gaussian distributions for many Vβ-chain repertoires had developed. In the $CD8^+$ T cells, mainly oligoclonal or polyclonal populations were present at 5 months post gene therapy, perhaps indicating prior antigen stimulation. However, at 15 months post gene therapy several Vβ families displayed normal patterns. Hence, at 15 months there was a broadly diversified T cell repertoire in both $CD4^+$ and $CD8^+$ cells (Figure 5.8).

5 months post gene therapy



15 months post gene therapy

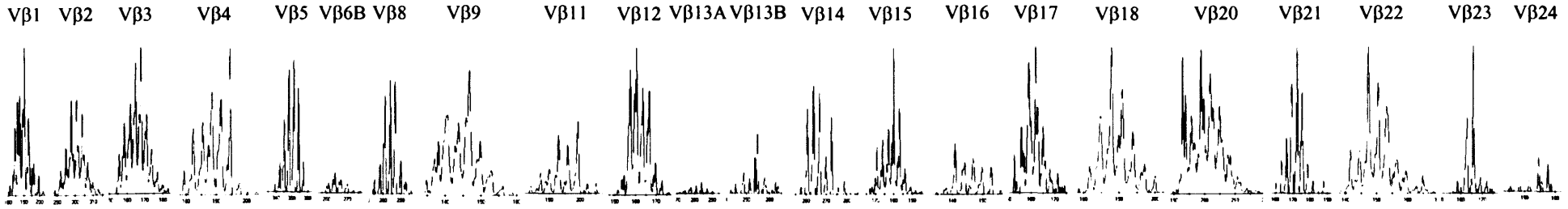
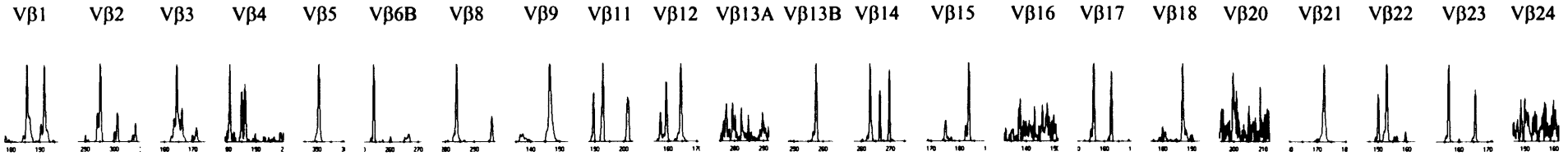


Figure 5.8A. CD4 spectratypes for Patient 3. Patient blood samples were collected at regular intervals and $CD4^+$ cells were separated using magnetic beads columns. The RNA was extracted and reverse transcribed. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. A run-off PCR was then performed and the CDR3 lengths were determined using a MegaBase sequencer machine.

5 months post gene therapy



15 months post gene therapy

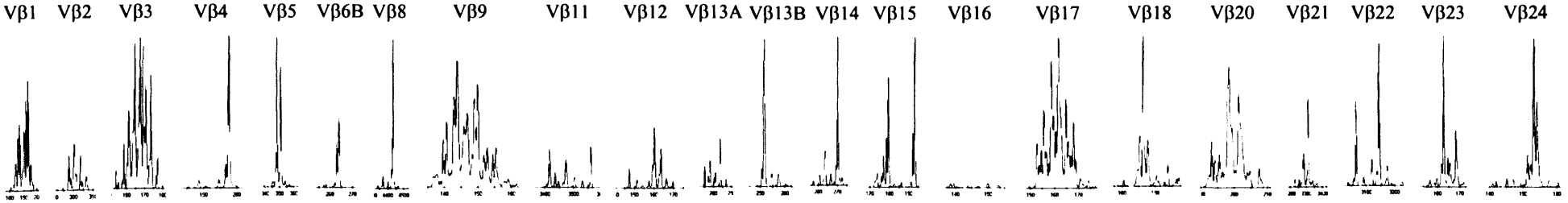


Figure 5.8B. CD8 spectratypes for Patient 3. Patient blood samples were collected at regular intervals and $CD8^+$ cells were separated using magnetic beads columns. The RNA was extracted and reverse transcribed. PCRs were performed on the samples using 24 different Vβ primers and one constant primer. A run-off PCR was then performed and the CDR3 lengths were determined using a MegaBase sequencer machine.

5.3 DISCUSSION

Patient 3 was treated with PEG-ADA therapy for three years, as no matched bone marrow donor was found. However, the immune function of patient 3 was clearly deteriorating, as indicated by his low lymphocyte counts and lack of thymic function. As his prognosis was poor, this patient was enrolled in the gene therapy study, and his immune recovery was examined.

Following gene therapy, patient 3 had a reactivation of a previous EBV infection. It is likely that with the removal of PEG-ADA and with the conditioning in preparation for gene therapy, the EBV infection was allowed to propagate. As it was persistent and difficult to clear, the patient was treated with Rituximab, an anti CD20 antibody that depletes the B cell population where EBV resides, which resulted in a controlled infection. Thus, in several methods of analyses shown the B cells were absent approximately 3 months to 6 months post gene therapy.

A similar gene therapy protocol to the one described by Auiti *et al.* (2002a) was used to treat patient 3. PEG-ADA was discontinued four weeks prior to gene therapy, and the patient received a mild non-myeloablative conditioning 2 days prior to the reinfusion of the cells. The removal of PEG-ADA was important as it should create a selective advantage to the transduced cells, and the space generated by the conditioning should allow for the engraftment of an increased number of gene modified cells. The bone marrow cells were selected for $CD34^+$ cells which were transduced in a five day procedure in the presence of Retronectin. Patient 3 received $13x10^6$ cells/kg, of which 25-35% were transduced. The ADA^+ cells had an average copy number of 2.33., which corresponds well to previously published results by Kustikova *et al.* (2003). They found an average of 1.6 vector insertions per transduced cell for a gene transfer efficiency of less than 31.6%, 2 for 42.5% and 3 for 52.8% in $CD34^+$. It is important to note that the copy numbers of the transduced cells were low as they were in the range of 1-4 copies per cell. Low copy numbers in the transduced cells reduce the risk of insertional mutagenesis, which is especially significant in the light of the recent leukaemia cases in the Paris X-SCID trial (Hacein-Bey-Abina *et al.*, 2003) (further discussed in Chapter 8).

Following gene therapy, the transgene copy number in B cells, T cells, NK cells, mononuclear cells, granulocytes and $CD34^+$ cells was estimated using real time quantitative PCR. The transgene was present in all cell types analysed up to 16 months post gene therapy and the highest level of transgene copy was found to be in T cells and NK cells with an average level of 0.5 and 0.4 copies per cell respectively, 16 months post gene therapy. A copy number of less than one implies that not all of the cells are transduced. Thus there must be a general detoxification in the haematopoietic system to allow for the survival of ADA^- cells. Aiuti *et al.* (2002a) found slightly higher levels of transduced cells in their two patients, but similarly to patient 3, it was the T cells and NK cells which showed the highest level of transduction as they were all transduced.

Most of the lymphocyte counts, apart from CD19 cells, have recovered following the withdrawal of PEG-ADA and stabilised at levels which are slightly higher to pre gene therapy. However, they have clearly not improved to the same extent as the lymphocyte counts for patients 1 and 2 who are receiving PEG-ADA. However, the concentration of the metabolite dATP reveals that although the lymphocyte counts are not normal for patient 3, there are enough ADA positive cells post gene therapy to maintain the dATP concentration at a level similar to post-bone marrow transplantations. Thus, the level of transduced cells and the level of ADA transgene expression can keep the haematopoietic system detoxified, which is corroborated by the results obtained for transgene copy numbers.

Encouraging results regarding generation of naïve cells also ensued following gene therapy. It was shown that the low proportion of $CD45RO^-CD27^+$ cells increased 2 months following gene therapy and has risen from 4.4% to 31.2% of $CD45^+$ cells in 9 months. A distinct population of $CD27^{high}$ cells has also developed, a good indication of truly naïve cells being produced. This suggests that some thymic function is recovering, supported by the detection of TREC activity for the first time 14 months post gene therapy (However, TRECs might have been expected to be detected earlier as shown by Talvensaari *et al.*, 2002.). An improvement in T cell diversity was also apparent at 15 months post gene therapy, as the spectratyping of the CDR3 revealed expected Gaussian distributions for many of the β-chain repertoires in both $CD4^+$ and $CD8^+$ cells.

Today, patient 3 is clinically well, free of infection and thriving. Significant immune reconstitution has occurred 15 months following gene therapy as indicated by the improvement of lymphocyte counts. The dATP concentration is also maintained at a low level, suggesting effective detoxification from gene therapy. The increase in $CD45RO^{-}CD27^{+}$ cells and detection of TREC activity also indicate that thymic function is improving. However, his immune reconstitution has clearly not been as good with neither PEG-ADA nor gene therapy, as that of patients 1 and 2 with PEG-ADA treatment. The reasons as to why patient 3 has not showed as good an immune recovery are not immediately clear. Based on the number of transduced cells returned, patient 3 was expected to show a similar immune reconstitution to patient 1 in the Aiuti study (2002a). Patient 1 in the Aiuti study received $8.6x10^{6}$ cells/kg, of which 25% were transduced, and patient 2 received only $1.08x10^{6}$ cells/kg of which 21% were transduced. Patient 1 in the Aiuti study has showed a good immune reconstitution, with the ALC reaching 3,500 cells/μL 20 months post gene therapy, however patient 2 did not reconstitute as well and has an ALC of only 400 cells/μL. Patient 3 in our study received $13x10^{6}$ cells/kg, of which 25% were transduced. However, at 14 months post gene therapy he has an ALC of only 800 cells/μL.

Therefore, although the immune function of patient 3 has clearly improved following gene therapy he has only showed a partial immune reconstitution, similar to that of patient 2 in the Italian study. Perhaps thymic recovery is compromised in older patients such as patient 3. It has been observed in mice that developmental arrest at early stages of T cell development results in severe thymic hyperplasia and thymic epithelial cell disorganisation (Hollander *et al.*, 1995; Van Ewijk *et al.*, 2000; Klug *et al.*, 2002). Similarly, ADA deficient mice show severe depletion of T lymphocytes and defects in T cell development as a result of metabolic abnormalities affecting various signalling pathways that regulate T cell survival and function. Therefore, there may be a time dependent limit on the successful re-initiation of thymopoiesis (Hollander *et al.*, 1995; Thrasher *et al.*, 2005) and the age of the patient may be an important factor in the success of gene therapy. Moreover, less damage may have been done at a younger age as a result of a lack of immune system and possibly as a result of infections.

Stem cells are the ultimate target cells of haematopoietic gene therapy as they have the ability to self-renew and can give rise to all cells of the immune system, therefore maintaining the expression of ADA in all haematopoietic cell types. However, a major disadvantage in using a gammaretrovirus is that it cannot infect quiescent stem cells. It is therefore necessary to activate the stem cells to divide, which usually results in differentiation. It has been previously demonstrated that extended culture of $CD34^+$ cells results in lower engraftment levels in mice (Bhatia *et al.*, 1997; Gothot *et al.*, 1998; Rebel *et al.*, 1999; Demaison *et al.*, 2000). During the gene therapy transduction procedure for patient 3 the percentage of $CD34^+$ cells was found to decrease, reducing the possibilities of transducing stem cells. In order to determine whether stem cells have been transduced integration site analysis can be performed. If several different cell types are found to have identical integration sites, then it is highly likely that they have originated from one transduced stem cell. Another assay that could be used is the traditional NOD-SCID mouse model, as engraftment of transduced cells in a secondary transplant would reveal the existence of transduced stem cells.

Hence it has been demonstrated that gene therapy is a good option when there is no matched bone marrow donor and when PEG-ADA is ineffective. The gene therapy trial of patient 3 can be considered a success as it was shown to be a well tolerated procedure, and patient 3 is now clinically well at home and thriving. He has been off PEG-ADA for more than one year and remains detoxified. As a result there has been an improvement in the immune function, although he still receives prophylactic immunoglubulins. A significant presence of gene modified cells has been detected in all cell types analysed 16 months following gene therapy. Continued follow-up of patient 3 will show whether he achieves complete functional recovery.

Based on the data obtained from the gene therapy trial, it will important to develop improved gene therapy protocols to treat ADA deficient patients. Reasons need to be identified as to why some previous ADA gene therapy trials have not been successful, such as perhaps the age of the patient at treatment and administration of PEG-ADA. It will also be essential to improve the transduction protocol to avoid the reduction in CD34 expression as seen during gammaretroviral transduction of $CD34^+$

cells. This could be done by identifying factors which maintain the progenitor cells and by decreasing the length of *ex vivo* culture of the $CD34^+$ cells.

6

CONSTRUCTION AND ASSESSMENT OF ADA LENTIVIRAL VECTOR

6.1 INTRODUCTION

Using a more efficient virus such as the lentivirus as the gene delivery vehicle in gene therapy trials would improve the transduction efficiency of stem cells and shorten the time of cell culture as the lentivirus has the ability of transducing non-dividing cells. Whereas gammaretroviruses rely on the breakdown of the nuclear membrane during mitosis, lentiviruses have developed a not yet fully elucidated mechanism of crossing the nuclear membrane to gain access to the host cell's genomic DNA into which the provirus integrates. This property of the lentivirus is particularly useful for the transduction of quiescent haematopoietic stem cells and slow dividing mesenchymal stem cells. The use of lentiviral vectors to transduce $CD34^+$ cells would reduce the time required for activation and the amount of cytokine dependence compared to gammaretroviral transductions, as lentiviruses only require minimal activation of stem cell division (Uchida *et al.*, 1998; Case *et al.*, 1999; Miyoshi *et al.*, 1999; Chang *et al.*, 1999). The reduced need for activation allows the $CD34^+$ cells to maintain their differentiation and engraftment potentials, thus maximising the possibility of haematopoietic repopulation in the patient. Hence, the use of lentiviruses may represent an improvement to the gene therapy protocol as it should result in high transduction efficiencies of both haematopoietic $CD34^+$ cells and mesenchymal stem cells.

The lentiviral vector used in our study is a second generation vector, whose env gene has been replaced by the surface glycoprotein of the vesicular stomatitis virus (VSV-G) (refer to figure 1.7 – backbone generated by Christophe Demaison). This envelope binds a receptor in the phospholipid bilayer of the cytoplasmic membrane of both vertebrates and invertebrates, and therefore confers a broad host range (Schlegel *et al.*, 1983; Marsh & Helenius, 1989). The pseudotyping of a lentiviral vector with VSV-G therefore alters its tropism from $CD4^+$ T cells and macrophages, to a broader host range. Importantly, pseudotyping with VSV-G also increases the stability of the viral particle, enabling ultracentrifugation and repeated freeze-thaw cycles without the loss of viral titre (Burns *et al.*, 1993; Ory *et al.*, 1996). A clear advantage of the ability to concentrate the virus pseudotyped with VSV-G is that one can use a high MOI for transduction. However, VSV-G has been associated with some cytotoxicity due to its fusogenic properties, limiting the concentration of vector

that can be used. This toxicity has also hindered the development of lentiviral packaging cell lines that pseudotype the vector with VSV-G.

The lentivirus used in this study has the safety feature of being a self-inactivating (SIN) vector where a 400 base pair deletion has been introduced in the U3 region of the 3' LTR used to produce the vector RNA (Zufferey *et al.*, 1998). During reverse transcription this deletion is transferred to the 5' LTR, resulting in the creation of an inactive 5' LTR which prevents the synthesis of full-length vector RNA in transduced cells, and the expression of the transgene is instead driven by the internal promoter. This reduces the number of active promoters from two (the 5' and 3' LTRs) to one (the internal promoter). The introduction of this mutation also eliminates the LTR promoter activity without affecting the viral transduction efficiency or transgene expression *in vitro*. SIN vectors with internal promoters, instead of LTR driven vectors, are thought to reduce the risk of activating neighbouring genes, thus minimising the risk of insertional mutagenesis. It is also thought that the risk of producing replication competent lentivirus will be reduced. Hence this modification is thought to be another step to achieve greater biosafety of the lentiviral vector.

The lentivirus used contained the cPPT element, important in the process of crossing the nuclear membrane, thus increasing the transduction efficiency of the virus by its ability to transduce quiescent cells (Zennou *et al.*, 2000; Sirven *et al.*, 2000; Demaison *et al.*, 2002; Manganini *et al.*, 2002). Similarly to the gammaretroviral vector, the lentivirus also carried the SFFV promoter and the WPRE.

Therefore, the principal aim was to construct an ADA lentiviral vector, to test its efficiency in different cell types and to ensure that the lentiviral mediated expression of ADA transgene does not have a toxic effect on the cells. Once this had been confirmed, the lentiviral vector was used to transduce $CD34^+$ cells and in the following chapter to transduce mesenchymal stem cells as possible target cells for gene therapy of ADA deficiency.

6.2 RESULTS

6.2.1 Generation of Lentiviral Vectors

The lentiviral construct, pHR.sin.cPPT.SAW, was generated, incorporating the SFFV promoter, human ADA cDNA and the WPRE (Figure 6.1A). The pBS-SAW vector was digested with *EcoRI* and *KpnI* to release the SAW fragment, which was inserted into the lentiviral backbone.

The eGFP-lentiviral DNA construct (pHR.sin.cPPT.SEW) was kindly provided by Dr Kate Parsley, ICH. It consisted of the same backbone and elements as the ADA-lentivirus, but rather than encoding ADA, it encoded the eGFP transgene (Figure 6.1B).

Lentivirus was generated by the transient transfection of 293T cells. The viral supernatant was harvested 48 and 72 hours following the transfection, and was then titred on HeLa cells in a similar manner to how the gammaretrovirus was titred. The viral titre for ADA-lentivirus was approximately $2x10^8$ transducing units/ mL, and for eGFP $1x10^9$ transducing units/mL.

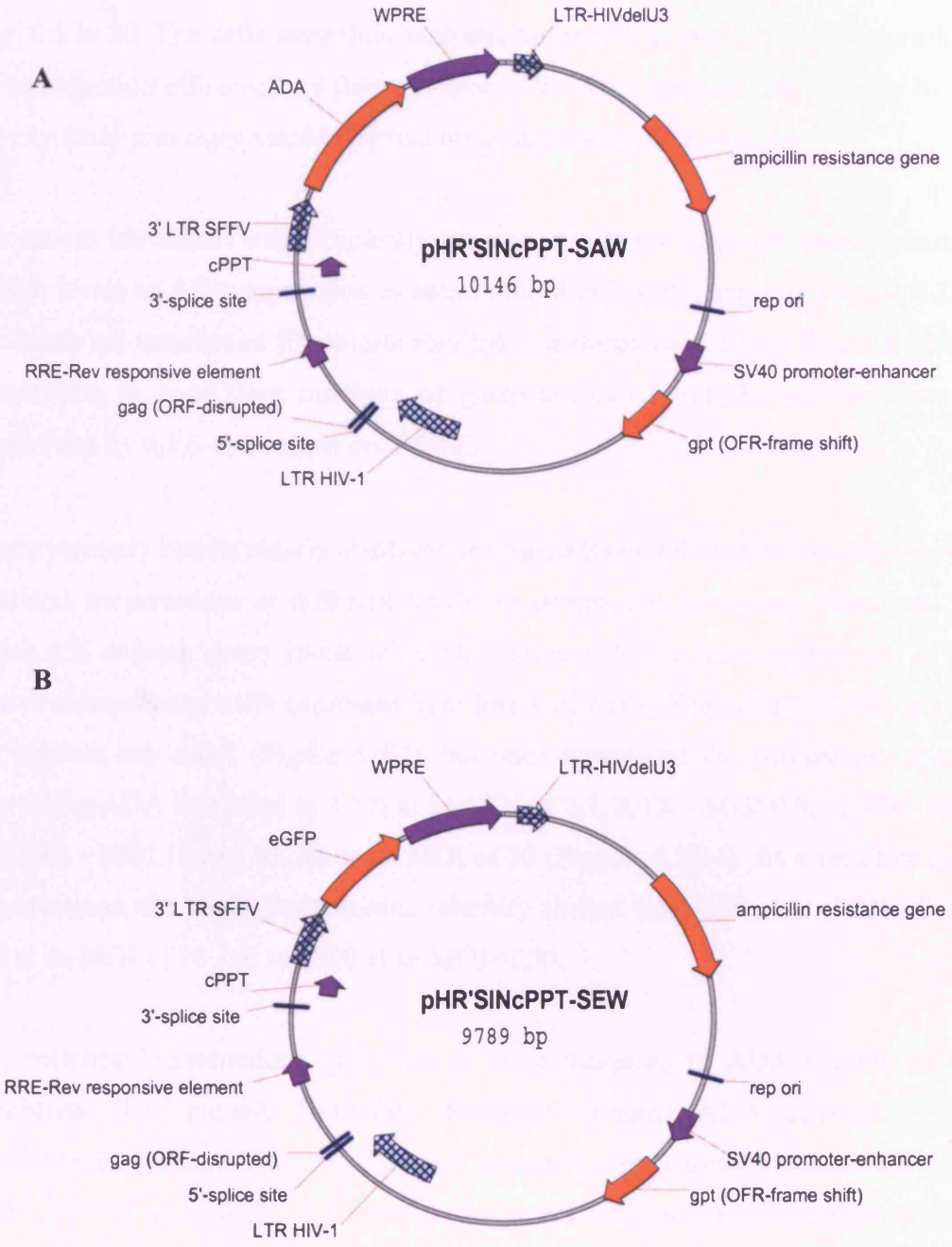


Figure 6.1. Lentiviral constructs.

A, Vector map of the lentiviral ADA construct. The lentiviral vector incorporates the SFFV promoter driving the transcription of ADA, whose expression levels are enhanced by the WPRE; **B,** Vector map of the lentiviral eGFP construct. This vector, similarly to the ADA lentiviral vector, carries the SFFV promoter and the WPRE element.

6.2.2 Lentiviral Transduction of Patient Fibroblasts

To test the efficiency of the lentiviral vector, patient primary skin fibroblasts were transduced with ADA-lentivirus in one round of transduction, using an MOI ranging from 0.1 to 30. The cells were then analysed for protein expression by Western blot, for transduction efficiency by flow cytometry, for ADA enzyme functionality by the activity assay and copy number by real time quantitative PCR analysis.

The patient fibroblasts were efficiently transduced with the lentiviral vector, resulting in high levels of ADA expression as seen in the Western blot analysis (Figure 6.2A). The lentiviral transduced fibroblasts resulted in a strong band in the Western blot in comparison to equivalent numbers of gammaretroviral transduced fibroblasts as determined by the β-actin expression levels.

Flow cytometry results clearly displayed the transduction efficiencies obtained by the lentiviral transductions at different MOIs. In comparison to normal fibroblasts, of which 1% express some levels of ADA (Figure 6.2C), a large proportion of the lentiviral transduced cells expressed high levels of ADA. The patient fibroblasts did not express any ADA (Figure 6.2D), but once transduced the percentage of cells expressing ADA increased to 3.1% at an MOI of 0.1, 8.3% - MOI 0.5, 42.1% - MOI 1, 50.6% - MOI 10 and 95.2% at an MOI of 30 (Figures 6.2E-I). As a result of these transductions, the mean fluorescence intensity shifted from 130 at an MOI of 1 to 310 at an MOI of 10 and to 3300 at an MOI of 30.

The lentiviral transductions gave rise to large increases in ADA activity of the fibroblasts. The patient fibroblasts displayed minimal ADA activity – 14.3 nmoles/hr/mg protein. This activity level increased greatly to 6,017 at the low MOI of 0.1, 17,701 at an MOI of 0.5 and to 3,824,521 nmoles/hr/mg total protein at an MOI of 30 (Figures 6.3A and B). These values are very high in comparison to normal fibroblast activity of 1,756 nmoles/hr/mg total protein. Lentiviral transduction therefore resulted in activity levels several folds higher than that found in normal fibroblasts. At an MOI of 0.1, the activity levels were 3.4 times the amount of normal activity levels of fibroblasts, at an MOI of 0.5, 10 times the normal activity

levels and at an MOI of 30, more than 2000 times greater than normal activity levels of fibroblast.

As expected, the copy numbers (average number of ADA transgene copies per cell) observed in the transduced fibroblasts increased with the increase in MOI. Transductions at an MOI of 0.1 resulted in a copy number of 0.0258, transductions at an MOI of 0.5 resulted in a copy number of 0.0968, and transductions at an MOI of 30 resulted in a copy number of 8.49 (Figure 6.3A). From these values one can determine the average level of enzyme activity produced per transgene copy. This was found to be approximately 200,000 nmoles/hr/mg protein per copy for the MOIs of 0.1 and 0.5, but as much as 450,000 nmoles/hr/mg protein per copy for the MOI of 30 (Figure 6.3A).

Therefore, the lentiviral construct generated in this study efficiently transduced fibroblasts and reconstituted ADA expression and activity.

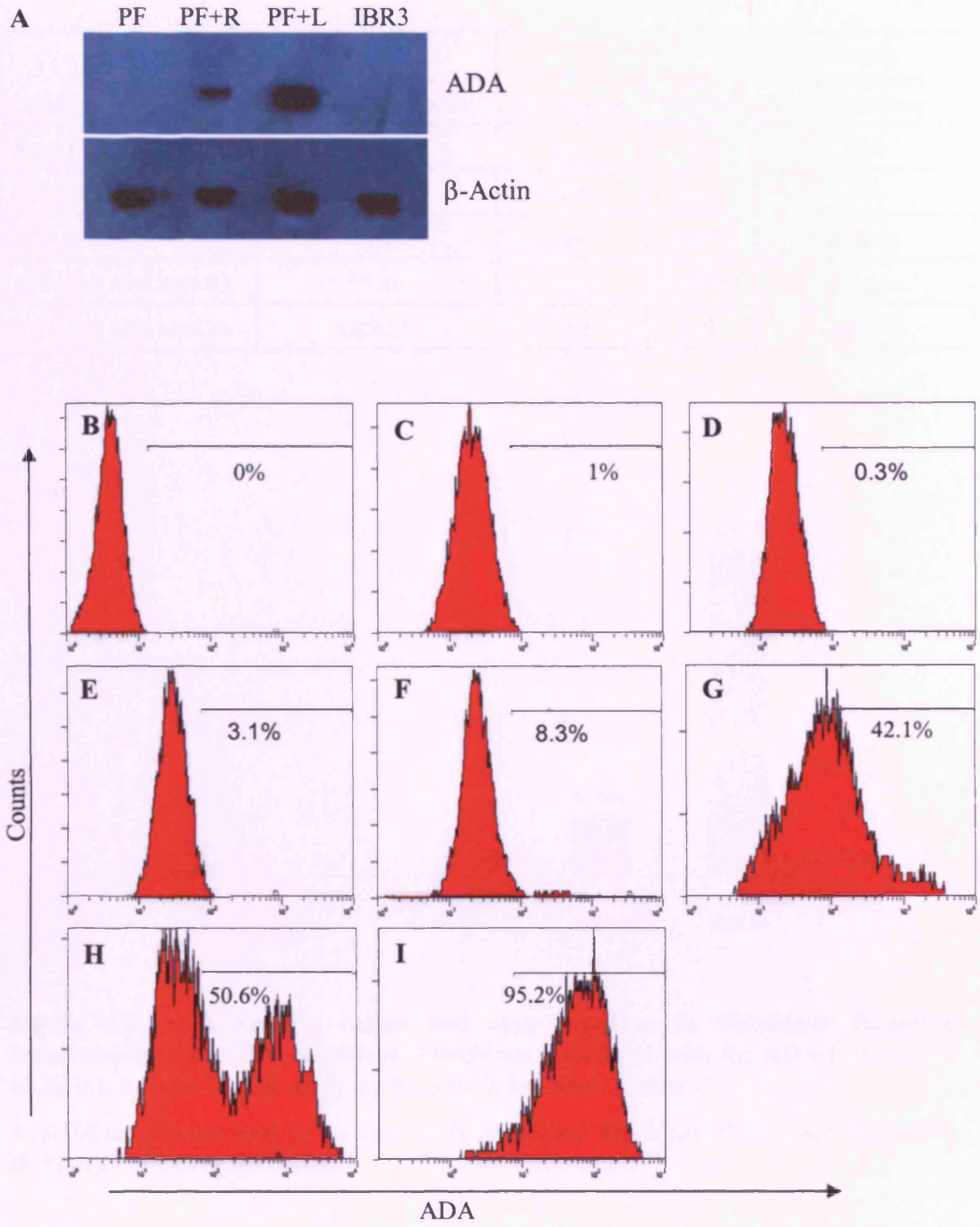


Figure 6.2. Lentiviral transductions of fibroblasts. The patient fibroblasts were transduced with the ADA lentivirus at MOIs ranging from 0.1 to 30.

A, Western blot depicting untransduced fibroblasts (PF), gammaretroviral transduced patient fibroblasts (PF+R), lentivirally transduced patient fibroblasts MOI 30 (PF+L) and normal fibroblasts (1BR3). **B-H,** Flow cytometry plots depicting the transduction efficiency of the lentiviral vector at MOIs ranging from 0.1 to 30. **B**, Secondary antibody only control; **C**, normal fibroblasts; **D**, patient fibroblasts; **E,** MOI 0.1; **F**, MOI 0.5; **G**, MOI 1; **H**, MOI 10; **I**, MOI 30. (The values in the flow cytometry plots refer to the ADA^+ cells).

A

Cells	ADA activity (nmoles/hr/mg protein)	% Transduction	Copy Number	Activity (nmoles/hr/mg protein)/Copy
Norm F	1,756	N/A	N/A	N/A
Patient F	14.3	N/A	N/A	N/A
P F + lenti ADA MOI 0.1	6,017	3.1	0.0258	233,232
P F + lenti ADA MOI 0.5	17,701	8.3	0.0968	182,865
P F + lenti ADA MOI 30	3,824,521	97.2	8.49	450,633

B

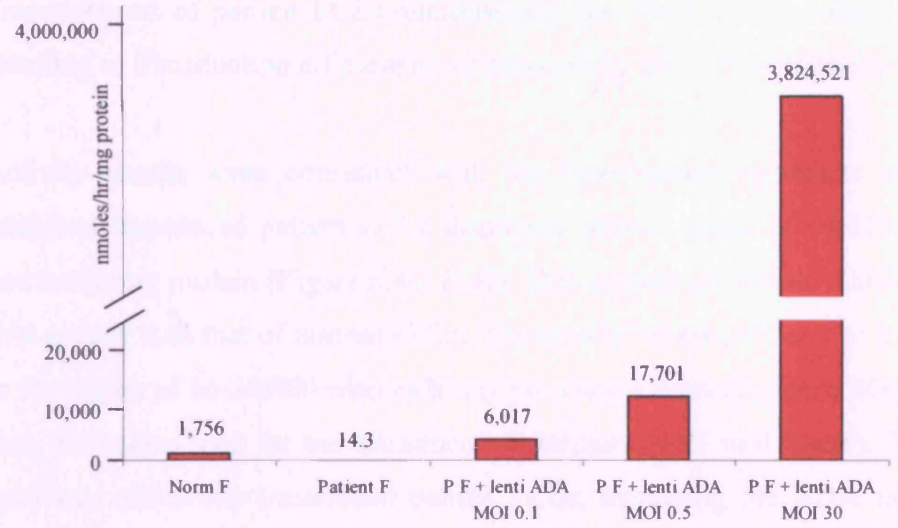


Figure 6.3. ADA activity values and copy numbers in fibroblasts following transductions with ADA-lentivirus. Fibroblasts transduced with the ADA-lentivirus at MOIs 0.1, 0.5 and 30 were analysed for activity and copy number.

A, ADA activity per copy of the lentivirally transduced fibroblasts. (N/A – not applicable)
B, Activity values of fibroblasts.

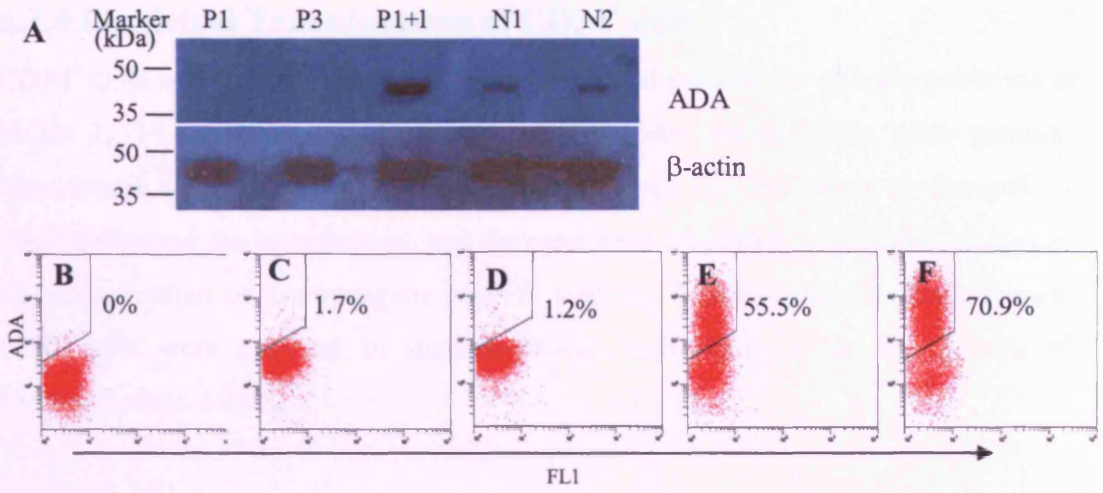
6.2.3 Lentiviral Transductions of Patient B-LCLs

LCLs were generated from blood samples of patients 1 and 3. The cells were transduced with the lentiviral vector, using an MOI of 15 in one round of transduction, without the use of fibronectin or polybrene. The transduced cells were then analysed for protein expression, copy number and activity levels.

The Western blot analysis revealed a large increase in ADA expression of lentiviral transduced patient 1 LCLs. In comparison, two normal LCLs showed only low levels of ADA expression from an equivalent amount of cells as determined by the β-actin expression (Figure 6.4A).

Transductions of patient LCLs demonstrated that the lentivirus was very effective, resulting in transduction efficiencies of between 55 and 70% (Figures 6.4E and F).

Activity results were consistent with the flow cytometry results obtained. The lentiviral transduced patient LCLs displayed activity levels of 64,017 and 155,430 nmoles/hr/mg protein (Figure 6.4G & H). This is equivalent to an ADA activity 7-20 fold greater than that of normal LCLs. The activity levels per copy of transgene were in the region of 16-30,000 nmoles/hr/mg protein per copy (Figure 6.4G), much lower than the values seen for the transduced fibroblasts (6-27 fold lower). Therefore, the lentivirus efficiently transduced patient LCLs, increasing the ADA expression and activity.



G

Cells	ADA activity (nmoles/hr/mg protein)	% Transduction	Copy Number	Activity (nmoles/hr/mg protein)/Copy
Normal LCLs KG	6,641	N/A	N/A	N/A
Normal LCLs EB	8,799	N/A	N/A	N/A
Patient 3 LCL	374	N/A	N/A	N/A
Patient 3 LCL + retro ADA	3,594	3.5	0.09475	37,891
Patient 3 LCL + lenti ADA	64,017	55.5	3.870	16,542
Patient 1 LCL	313	N/A	N/A	N/A
Patient 1 LCL + lenti ADA	155,430	70	5.09	30,536

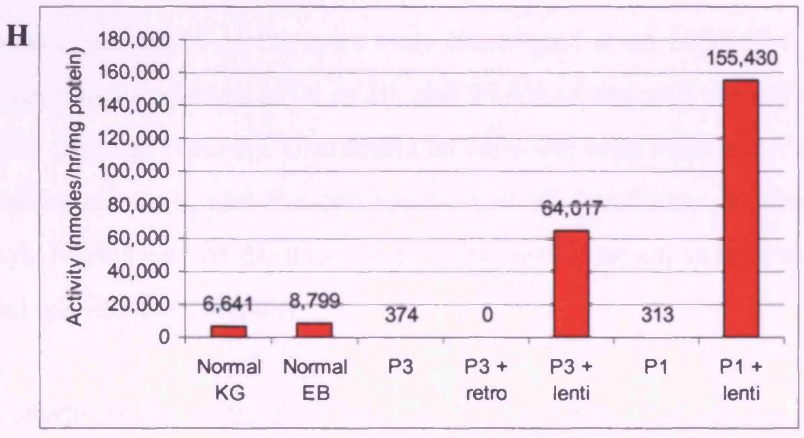


Figure 6.4. Lentiviral transduction of patients' B-LCLs. LCLs were transduced with the ADA lentivirus at an MOI of 15.

A, Western blot depicting ADA expression in LCLs. P1 and P3, patient LCLs; P1+l, lentiviral transduced patient LCLs; N1 and N2, normal LCLs.

B-F, Flow cytometry plots displaying the transduction efficiency of lentiviral transduction of patient LCLs. **B,** Secondary antibody only control; **C**, Normal LCLs; **D,** untransduced patient 3 LCLs; **E**, lentiviral transduced patient 3 LCLs; **F**, lentiviral transduced patient 1 LCLs. (The values in the flow cytometry plots refer to the ADA+ cells).

G, Activity values and copy numbers for the two patient LCLs. (N/A – not applicable)

H, ADA activity values of the transduced patient LCLs in comparison with normal LCLs.

6.2.4 Lentiviral Transductions of $CD34^+$ cells

$CD34^+$ cells were cultured in activation medium and transduced with the lentivirus at MOIs 1, 10 or 30 without pre-activation. Unlike transductions with gamma-retroviruses, no fibronectin or Retronectin was used. The medium was changed 24 hours following the transduction, and the cells were incubated for a further 2 days to allow expression of the transgene prior to analysis. Following the medium change, 1000 cells were cultured in methylcellulose medium to allow the growth of haematopoietic colonies.

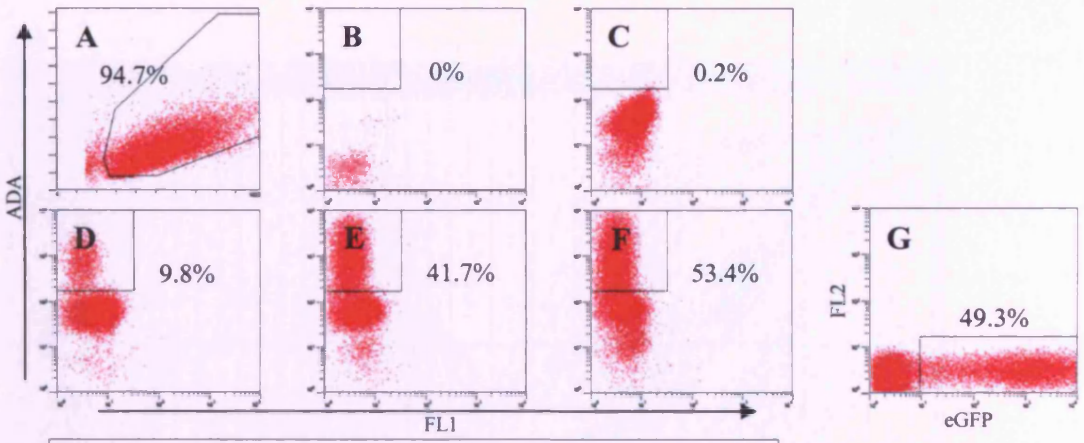
eGFP lentivirus was used as a control vector to assess gene transfer efficiency of $CD34^+$ cells. Flow cytometry analysis revealed that the cells were 49.3% transduced using an MOI of 30 (Figure 6.5G) and no toxicity was observed as determined by trypan blue analysis (data not shown). The lentiviral-eGFP transduced $CD34^+$ cells were cultured in methylcellulose medium, and the percentages of eGFP positive colonies observed were as follows:
Erythroid cells: 53% transduced
Myeloid cells: 26% transduced

ADA lentivirus was then used to transduce $CD34^+$ cells. Flow cytometric analysis showed that 9.8% of the cells were transduced at an MOI of 1, 41.7% of the cells were transduced at an MOI of 10, and 53.4% of the cells were transduced at an MOI of 30 (Figures 6.5D-F). Untransduced cells and cells transduced at the varying MOIs proliferated well, and the cell numbers at all conditions doubled or tripled in four days. No toxicity of the transduced cells was observed, as determined by trypan blue analysis (data not shown).

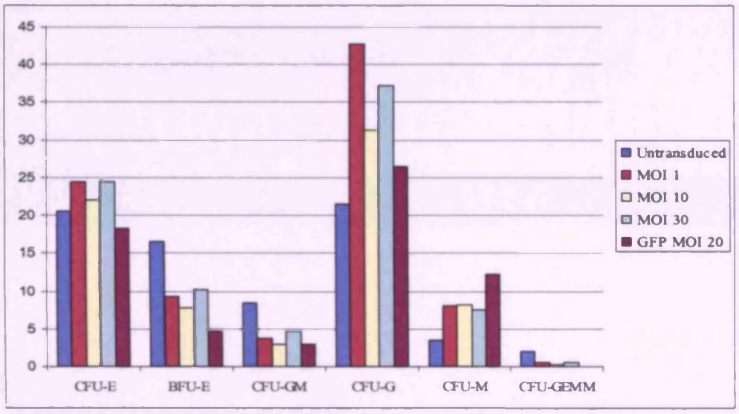
Analysis of the methylcellulose colonies revealed that similar proportions of each colony type were observed for cells of each transduction condition and untransduced cells (Figure 6.5H). However this experiment, which was only performed once, should be repeated to confirm that the progenitor cell phenotype has not been altered by the transduction procedure. It was found by PCR that at an MOI of 1, 1 CFU-E colony was positive for the ADA transgene. An MOI of 10 gave rise to 2 CFU-G, 3 CFU-M, 1 CFU-GM, 2 CFU-E and 3 BFU-E transduced colonies. An MOI of 30

gave rise to 3 CFU-G, 4 CFU-M, 4 CFU-GM, 4 CFU-E and 2 BFU-E transduced colonies (Figure 6.5I).

Real-time PCR analysis was performed on colonies grown from ADA-lentiviral transduced $CD34^+$ cells (Figures 6.6A and B). Average copy number per cell was calculated using β-actin as a standard. The range of copy numbers and the average copy number of the transduced colonies increased with increasing MOIs. The following copy numbers were observed: cells transduced at an MOI of 1 had an average copy number of 1.5, cells transduced at an MOI of 10 had a range of copy numbers of 1-10, and an average copy number of 3.77, and cells transduced at an MOI of 30 had a range of copy numbers of 1-18, and an average copy number of 5.89 (Figure 6.5J).



H



I

Colony type	MOI 1	MOI 10	MOI 30	Retro
CFU-G		2	3	1
CFU-M		3	4	
CFU-GM		1	4	
CFU-E	1	2	4	2
BFU-E		3	2	3

J

Transduction efficiencies	MOI 1	MOI 10	MOI 30	Retro
Average copy number of positives	1.52	3.77	5.89	2.33
Range of copy numbers	1.52	1-10	1-18	1-4
% positive by FACS	9.8	41.7	53.4	25.2
% positive by PCR of mc	5	55	85	30

Figure 6.5. Lentiviral transductions of CD34⁺ cells. CD34⁺ cells were activated by a cytokine mix and transduced with ADA or eGFP lentivirus at MOIs ranging from 1 to 30.

A-G, Flow cytometry plots displaying the transduction efficiency of the lentiviral vector at different MOIs. **A-F**, ADA-lentiviral transduced cells. **A**, forward scatter side scatter plot reveals the viable population of cells which is gated; **B**, secondary antibody only control; **C**, untransduced CD34s; lentiviral transduced CD34s at an MOI of **D**, 1; **E**, 10; and **F**, 30. (**A-F:** The values in the flow cytometry plots refer to the ADA⁺ cells). **G**, Lenti-eGFP transduced cells, MOI 30 (The values in the flow cytometry plot refers to eGFP⁺ cells). .

H, Numbers of colony types for the different transduction conditions. For each condition, 1000 cells were cultured in methylcellulose.

I, Types of colonies transduced by the lentivirus.

J, Copy numbers of the transduced colonies, assessed by real time quantitative PCR; mc, methylcellulose colonies.

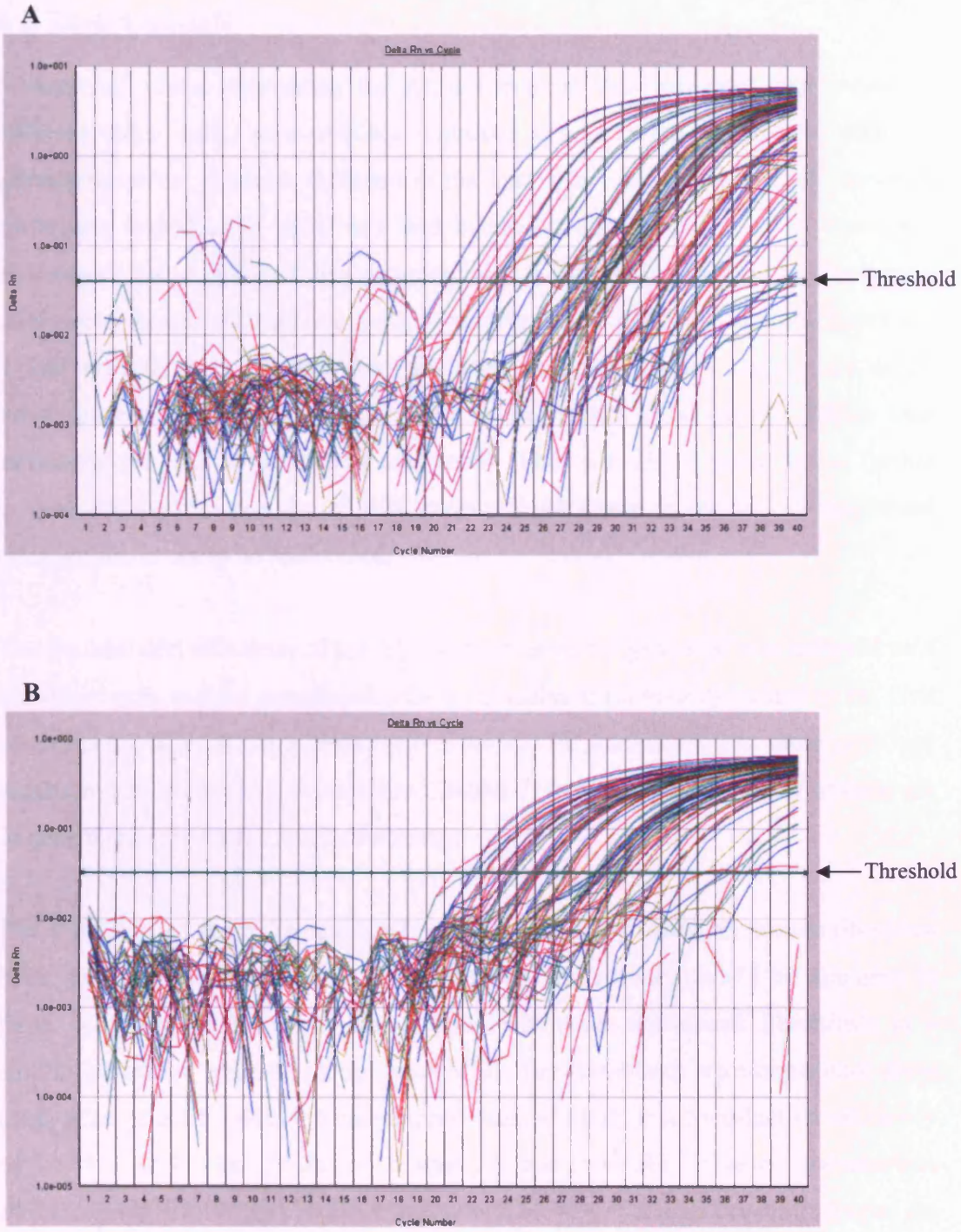


Figure 6.6. Real time PCR analysis of lentiviral transduced CD34⁺ cells. Transduced CD34⁺ cells were analysed for **(A)** the ADA transgene and **(B)** for β-actin.

6.3 DISCUSSION

A lentiviral vector expressing the ADA transgene was generated and tested on different target cells, as a possible improved gene transfer vehicle. As with the gammaretrovirus, elements included in the lentivirus were selected on the basis of optimising transduction efficiency and achieving high levels of ADA transgene expression. The ADA-lentivirus generated was a SIN vector, thought to represent a safer vector system as there are reduced numbers of functional promoters. However, it has recently been shown that the lentiviral SIN vectors display as much transcriptional read-through as gammaretroviral vectors (Zaiss *et al.*, 2002), thus increasing the risk of activating oncogenes. This will clearly need to be further investigated prior to the use of SIN vectors in the clinic as the risk of insertional mutagenesis needs to be minimised.

The transduction efficiency of the ADA-lentiviral vector generated was assessed on a variety of cells and the transduced cells were analysed for toxicity related to the viral transduction. The ultimate target cells were $CD34^+$ haematopoietic stem cells and mesenchymal stem cells (discussed in Chapter 7) to assess the possibility of their use in gene therapy trials for ADA deficiency.

The efficiency of the ADA-lentiviral vector was initially tested on patient fibroblast cells, as the reconstitution of ADA expression and activity should be apparent in these ADA null cells. Gammaretro- and lentiviruses transduced fibroblasts at a similar efficiency when using the same MOI. The fibroblasts were transduced three times at an MOI of 1 with the gammaretrovirus, resulting in a transduction efficiency of 21.4%. At similar MOIs of 1 and 10, the lentivirus achieved transduction efficiencies of 42.1% and 50.6% respectively. However, a high concentration of the gammaretrovirus could not be obtained as the GALV envelope does not allow ultracentrifugation, thought to be due to structural characteristics of the envelope protein. In contrast, the VSV-G envelope confers stability to the viral particle, allowing the lentivirus to be concentrated by this method so that high MOIs can be achieved. Hence using the lentivirus at an MOI of 30, 95.2% of the cells were transduced. The MFI also increased significantly with transductions at high MOIs.

It is important for the treatment of ADA deficient patients that the expression of ADA is maintained as it is an enzyme which is ubiquitously expressed throughout life. ADA expression was therefore measured by flow cytometry in both the gammaretroviral and lentiviral transduced patient fibroblasts shortly after transduction and after a period of time. Both gammaretroviral and lentiviral transgene expressions were found to be stable as the percentage of ADA expressing cells remained the same following 6 months of culture (data not shown).

The activity values of the transduced fibroblasts were found to be very high. Normal fibroblasts had an activity of 1,765 nmoles/hr/mg protein. Gammaretroviral transductions resulted in an activity value of 30,743 nmoles/hr/mg protein, 17-fold that of normal fibroblasts. Lentiviral transductions at MOIs 0.1 (3.1% transduced), 0.5 (8.3% transduced) and 30 (95.2% transduced) resulted in activities of 6,017 nmoles/hr/mg protein, 17,701 nmoles/hr/mg protein, and 3,824,521 nmoles/hr/mg protein respectively. These activity values are equivalent to 3-fold, 10-fold and over 2000-fold the activity levels of normal fibroblasts. Although fibroblasts are not the ultimate target cells in gene therapy, these results suggest that low MOIs with lentiviral vectors should be sufficient to restore ADA expression and activity in patient cells. The activity in lentiviral transduced cells per transgene copy was found to be in the region of 200-400,000 nmoles/hr/mg protein per copy, similar to that of the gammaretroviral transduced fibroblasts which had a value of nearly 200,000 nmoles/hr/mg protein per copy. Thus, each copy of gammaretroviral and lentiviral vector results in similar amounts of ADA activity.

The greater gene transfer efficiency of the lentiviruses compared with gammaretroviruses was made apparent by the transductions of patient LCLs. Gammaretroviral transductions of LCLs resulted in very low transduction efficiencies in the region of 5%, using an MOI of 1 for each of the three rounds of transductions and the assistance of both fibronectin and polybrene. However, lentiviral transductions of the same cells gave rise to 60-70% transduced cells using an MOI of 15 in one round of transduction and no fibronectin or polybrene. These transduction efficiencies appear to be higher than published figures of 17-31% using an MOI of 5-10 (Bovia *et al.*, 2003).

The expression of ADA in lentiviral transduced LCLs was again importantly found to be stable as LCLs grown for more than three months showed the same percentage transduction as determined by flow cytometry.

Lentiviral transductions resulted in vast increases of ADA activity of patient B-LCLs from approximately 300 to 64,017-155,430 nmoles/hr/mg protein. This corresponded to activity levels 7-20 fold greater than the levels observed in normal LCLs. Hence, lentiviral transductions reconstituted ADA expression and activity in patient B cells which should be sufficient to eliminate the toxic ADA substrates of these cells.

The ADA activity per copy of transgene in the lentiviral transduced LCLs was found to be in the region of 15,000-30,000 nmoles/hr/mg protein per copy. These values are similar to those of the gammaretroviral transduced LCLs which had an activity of nearly 40,000 nmoles/hr/mg protein per copy. However, the activities per copy in the LCLs are significantly lower (6-27 fold lower) than those observed in the transduced fibroblasts, which were found to be 200,000-450,000 nmoles/hr/mg protein per copy. Thus, although the gammaretroviral and lentiviral-ADA have integrated into the genome of the LCLs they may not be expressing the ADA protein, or the ADA enzyme expressed does not exhibit any activity. Low levels of protein expression could be due to promoter shut-off in the LCLs. However, if it is the case that the enzyme is expressed but not functional, this could be as a result of different post-translational modifications in the different cell types.

Although lentiviral vectors can transduce quiescent haematopoietic progenitors (Miyoshi *et al.*, 1999; Woods *et al.*, 2000; Sirven *et al.*, 2001), they have been found to be more efficient at transducing $CD34^+$ cells which are in G_1 or actively cycling in G_2/S/M (Sutton *et al.*, 1999). Therefore, a cytokine mix is added to the cultured cells in order to induce proliferation and improve gene transfer efficiency. However, the concentrations of cytokines used are lower than those for the gammaretrovirus, and no pre-activation of the cells is required. The lentiviral vector efficiently transduced $CD34^+$ cells at MOIs lower than 30. An MOI of 1 resulted in 9.8% transduced cells, MOI of 10, 41.7% transduced cells and an MOI of 30 resulted in 53.4% transduced cells. These are similar to the transduction efficiencies observed with the LCL, and to the gammaretroviral transductions of $CD34^+$ cells, where three rounds of

transductions at an MOI of 1 resulted in a transduction efficiency of approximately 25%. It is therefore advantageous to use lentiviral vectors to transduce $CD34^+$ cells mainly for the reason that limited activation is needed so that the cells do not need to be cultured *ex-vivo* for long periods of time, and the CD34 expression should therefore remain high. Hence, this experiment needs to be repeated to assess the CD34 expression levels following lentiviral transductions, and NOD/SCID engraftment studies of gammaretroviral and lentiviral transduced $CD34^+$ cells would need to be carried out to confirm this hypothesis. Previous studies have demonstrated that the culture of $CD34^+$ cells with cytokines results in reduced repopulation ability of these cells (Bhatia *et al.*, 1997; Gothot *et al.*, 1998; Rebel *et al.*, 1999; Demaison *et al.*, 2000 & 2002). Thus, engraftment studies are necessary to confirm whether a shorter transduction procedure using the ADA lentiviral vector would be beneficial.

Methylcellulose haematopoietic colony assays of ADA-lentiviral transduced $CD34^+$ cells revealed that the transduction procedure did not affect the proportions of different progenitors as untransduced and transduced $CD34^+$ cells gave rise to similar numbers of each colony type. It was found by PCR for the ADA transgene that all cell types have been transduced, and thus the lentivirus shows no preferential transduction of a particular cell type. This lack of preferential transduction is advantageous as the ideal target cell is an earlier progenitor cell, capable of self-renewal and differentiation into all haematopoietic lineages.

Real time PCR analysis of methylcellulose colonies also gave rise to some very interesting results. As each colony is derived from a single cell, this type of analysis can estimate the actual number of transgene copies in one transduced cell. This analysis showed that with increasing MOI, not only did the percentage of transduced cells increase, but the average copy number per cell increased as well. This is likely to be due to the fact that the cells which are easily transduced have already been infected, and increasing the amount of virus simply results in the extra virus infecting the same easily transducible cells rather than infecting other cells which are perhaps not dividing. Therefore, increasing the MOI from 1 to 10 increased the average copy number from 1.52 to 3.77, and increasing the MOI to 30 further increased the average copy number to 5.89. Gammaretroviral transduction resulted in a similar copy number (2.33), using an MOI of 1 in three rounds of transduction. Therefore, a

maximum MOI of less than 10 should probably be used in gene therapy trials with lentiviral vectors, as many integrated copies in each cell is not desired as it may increase the chance of insertional mutagenesis.

It has therefore been demonstrated that the constructed ADA lentiviral vector efficiently transduces several different cell types resulting in high levels of ADA expression and activity likely to be sufficient for detoxification, in the absence of cytotoxicity caused by lentiviral mediated expression of ADA transgene.

Gammaretro- and lentiviral vectors both have several advantages as gene transfer vehicles for ADA gene therapy. They both have a packaging capacity large enough for the ADA transgene, they do not exhibit vector induced immune responses, and crucially are integrating vectors resulting in long-term expression of the transgene. Gammaretroviral vectors are not naturally human pathogens like the HIV-1 lentivirus, and did therefore not have as many problems to reach clinical trials. In fact, gammaretroviral vectors have been used extensively in many gene therapy trials, and as a result there is plenty of experience with the use of this vector. Packaging cell lines have been generated for gammaretroviral vectors, advantageous due to their ease of use and the consistent titre achieved, as well as the reduced risk of recombination to generate replication competent retrovirus. Packaging cell lines are currently also being developed for lentiviral vectors, however, this has been hindered by the toxicity of the VSV-G envelope to the producer cells. Inducible promoters were investigated so that the VSV-G would not be constitutively expressed in the cell line (Kafri *et al.*, 1999; Klages *et al.*, 2000; Ni *et al.*, 2005), however viral production could still only be maintained for a few days due to cytotoxicity. Alternative envelopes may therefore need to be studied, such as RD114, a feline endogenous retrovirus envelope. Similarly to VSV-G, pseudotyping with RD114 allows ultracentrifugation of the virus and in addition is stable in human serum. However, virus pseudotyped with RD114 cannot transduce murine cells and therefore limits its use in the laboratory for pre-clinical testing. Hence, one disadvantage with the use of lentivirus pseudotyped with VSV-G is that a packaging cell line has not been generated.

Therefore, both gammaretroviral and lentiviral vectors have several advantages for use in gene therapy. However, the efficiency of transduction of lentiviral vectors makes it particularly well suited for the transduction of several cell types, including the quiescent $CD34^+$ stem cells and mesenchymal stem cells which will be investigated in the following chapter.

7

Mesenchymal Stem Cell Gene Therapy for ADA Deficiency

7.1 INTRODUCTION

ADA-SCID is a multi-organ disease, resulting in immunodeficiency as well as non-immunological abnormalities. Non-immunological symptoms include skeletal abnormalities (Cederbaum *et al.*, 1976) and liver problems (Bollinger *et al.*, 1996) as well as neurological abnormalities (Hirschhorn *et al.*, 1980; Tanaka *et al.*, 1996; Rogers *et al.*, 2001; Albuquerque & Gaspar, 2004). Bone marrow transplantations and gene therapy using $CD34^+$ haematopoietic stem cells or peripheral blood T cells may result in improved immunological function without necessarily correcting the non-immunological symptoms. Hence, gene therapy using HSCs in combination with MSCs may be an alternative to the use of HSCs alone and could offer a more systemic treatment for ADA deficiency.

MSCs were isolated, and their multipotential nature was analysed prior to and subsequent to viral transductions, exploring the possibility of their use as therapeutic targets in clinical gene therapy trials. MSCs form part of the non-haematopoietic environment in the bone marrow, and are readily isolated by ficoll gradient centrifugation followed by plastic adherence. They display a fibroblast-like morphology, but have a different expression pattern to fibroblasts, which are mature mesenchymal cells not capable of differentiation. MSCs have been reported to differentiate into several different cell types of the mesenchymal and non-mesenchymal lineage, including adipocytes, osteocytes, chondrocytes, myocytes and neuronal cells (Piersma *et al.*, 1983 & 1985; Howlett *et al.*, 1986; Friedenstein *et al.*, 1987; Mardon *et al.*, 1987; Owen & Friedenstein, 1988; Keating *et al.*, 1990; reviewed by Caplan, 1991; Haynesworth *et al.*, 1992; Beresford *et al.*, 1992; Cheng *et al.*, 1994.; Rickard *et al.*, 1994; reviewed by Clark & Keating, 1995; reviewed by Prockop, 1997; Pittenger *et al.*, 1999). Therefore, as gene delivery vehicles, MSCs should be very useful in the treatment of ADA deficiency by offering a systemic delivery of ADA. Hence, it is hoped that the homing of the ADA^+ MSCs to the areas of need in the body, and the differentiation of the ADA^+ HSCs into all cells of the haematopoietic system, could lead to a more complete treatment for ADA deficiency than previous gene therapy trials using T cells or $CD34^+$ cells alone. Moreover, MSCs have been reported to provide support for the haematopoietic stem cells upon transplantation (Dexter & Testa, 1976; Dexter *et al.*, 1977; Gartner & Kaplan, 1980;

Eaves *et al.*, 1991; Otsuka *et al.*, 1991; Kittler *et al.*, 1992; Quesenberry & Lowry, 1992; reviewed by Sutherland *et al.*, 1993; reviewed by Deryugina & Muller-Sieburg, 1993; Gordon *et al.*, 1996), which would be ideal in the case of gene therapy using both HSCs and MSCs. Majumdar *et al.*, 1998, observed that MSCs expressed cytokines that act on haematopoietic cells, maintaining and supporting differentiation of purified $CD34^+$ cells. Reports have also been published which show that co-transplantation of MSCs and HSCs improve haematopoietic engraftment following BMT in animal models (Nolta *et al.*, 1994 & 2002: Brouard *et al.*, 1998; Noort *et al.*, 2002; Bensidhoum *et al.*, 2004) and in humans (Koc *et al.*, 1999, 2000; reviewed by Koc & Lazarus, 2001). However, the exact identity of the cells capable of supporting HSCs is still being debated (reviewed by Minguell *et al.*, 2001). Therefore, as MSCs are thought to be a heterogeneous population of cells, more studies are required to assess the effect of isolation procedure and culture conditions on the growth of specific cell types, and to determine which cell type is best suited for engraftment and haematopoiesis support.

Therefore, the aim was to generate human MSCs and to confirm the multipotential nature of the cells. It is vital that the MSCs have a broad differentiation potential and that this remains following transduction. Therefore, the differentiation potential will be assessed prior to and following transduction with lentiviral vectors. It will be particularly important to establish whether the MSCs can differentiate into neurons, as it is hoped that neurological symptoms observed in ADA deficient patients can be improved by administering ADA-lentiviral transduced autologous MSCs. It will also be important to investigate the ability of MSCs to home to different organs in a xenotransplant animal model as this may predict how the MSCs will home in the patient. There are certain organs which are particularly important to target, including the liver and the brain which have displayed abnormalities in ADA deficient patients. Thus, the homing abilities of MSCs will be assessed in a NOD/SCID mouse model. This mouse was first used to assay human cell reconstitution by Lapidot *et al.* in 1992. In this model, the SCID mutation has been transferred onto a diabetes-susceptible Non-Obese Diabetic background. Animals homozygous for the SCID mutations experience a block in T and B-cell lymphocyte development (Schuler *et al.*, 1986) and they also have impaired NK cell and macrophage cell function, and lack complement activity (Schultz *et al.*, 1995). The multiple defects in immunity

unique to this model provide an excellent system for reconstitution with human haematopoietic and other cells.

7.2. RESULTS

7.2.1 Generation of Mesenchymal Stem Cells

MSCs were generated from bone marrow taken from the iliac crest of consenting patients or donors of less than 10 years of age. Bone marrow mononuclear cells (PBMCs) were isolated by density gradient (1.077g/mL Ficoll-Paque from Pharmacia), by centrifugation at 1,000xg for 20 minutes. These cells were then plated into a tissue culture flask, and MSCs were isolated by the plastic adherence method. Prior to passaging, it was possible to observe a large number of non-adherent cells (7.1A). Following three passages, the cells appeared homogeneous and displayed a fibroblastic morphology (Figure 7.1C). Cells used in any experiments would be of at least passage 3. The MSCs could usually be grown until passage 17, when senescence appeared to set in.

Once the MSCs had been generated, immunophenotyping was performed to confirm that they expressed markers previously reported for MSCs. Fibroblasts, a mature type of mesenchymal cells, were used as control cells and should have a slightly different phenotype compared to MSCs. Both MSCs and fibroblasts were of passage 5. The following markers were not detected on MSCs by flow cytometry: CD14, CD33, CD34 and CD45. Hence, they have a different expression pattern from HSCs also found in the BM, which express CD34 and CD45 (Table 7.1). They did express the following markers: CD13, CD29, CD44, CD73 (SH4), CD90, CD105 (SH2) and CD106 (Figure 7.2). Expression levels of certain markers were noticeably different from the fibroblasts. The MSCs expressed lower levels of CD44 compared to fibroblasts, and MSCs expressed CD106, which fibroblasts did not do (Table 7.1).

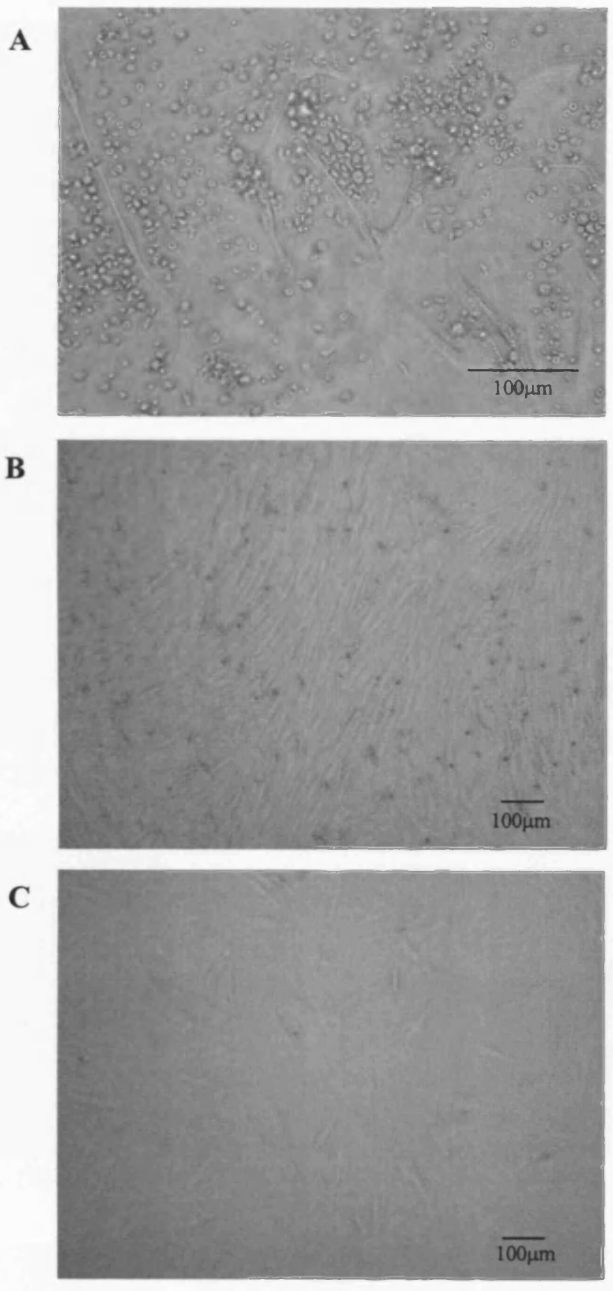


Figure 7.1. Phase contrast microscopy images of MSCs at passages 0, 2 and 3. Mesenchymal stem cells were isolated from bone marrow by ficoll density gradient centrifugation followed by plastic adherence. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A, Day 6 prior to passage; **B**, Passage 2; **C**, Passage 3.

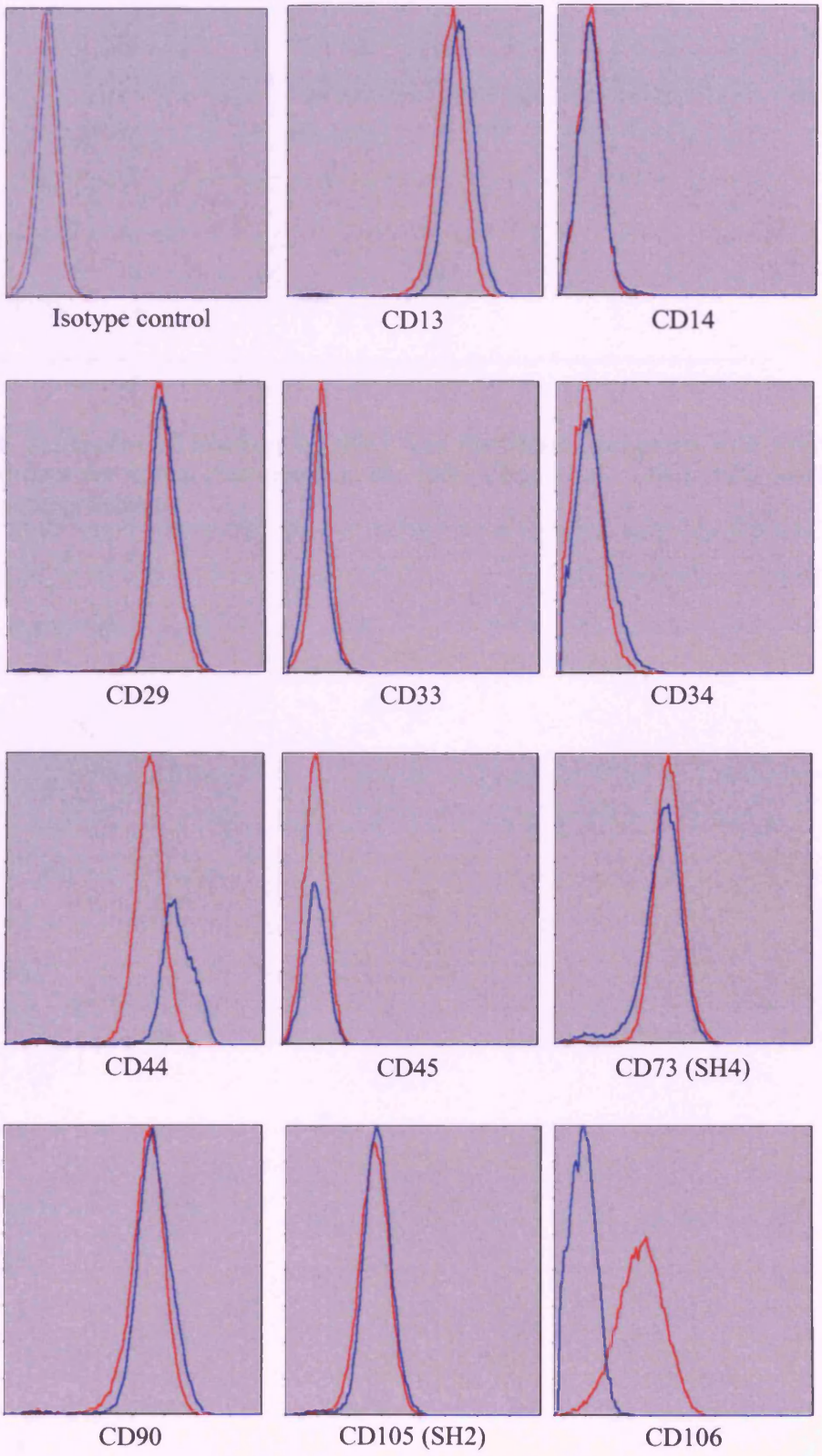


Figure 7.2. Normal MSC phenotype panel. Mesenchymal stem cells and primary normal fibroblasts both of passage 5 were stained with antibodies for different markers. The red graph depicts MSCs, the blue graph depicts fibroblast.

Marker	MSC P5	MSC P12	MSC SCC P12	Fibroblasts	HSC
CD13	+	+	+	+	-
CD14	-	-	-	-	-
CD29	+	+	+	+	-
CD33	-	-	-	-	-
CD34	-	-	-	-	+
CD44	+	+	+	++	-
CD45	-	-	-	-	+
CD73 (SH4)	+	+/-	+	+	-
CD90	+	+/-	+/-	+	-
CD105 (SH2)	+	+	+	+	-
CD106	+	-/+	-/+	-	-

Table 7.1. Expression of markers in MSCs and fibroblasts compared with previously published data for HSCs (Sutherland *et al.*, 1989; Civin *et al.*, 1996). SCC, single cell clone; P, passage number.

7.2.2 Differentiations of MSCs

To verify the multipotential characteristics of the mesenchymal stem cells isolated, they were analysed for adipogenic, osteogenic, chondrogenic and neuronal differentiation potential. Normal or patient MSCs of between passage 5-12 were grown in the appropriate differentiation medium for up to three weeks. The differentiated cells were then analysed using specific assays to confirm that differentiation had occurred.

7.2.2.1 Adipocyte Differentiation

Normal and patient MSCs were grown in adipocyte induction media for up to three weeks. The MSCs displayed in Figure 7.3 are from a normal individual, and the ADA patient MSCs differentiated in the same manner (data not shown). After approximately one week, a change toward adipocyte morphology was observed. The differentiated MSCs displayed a typical flattened adipocyte-like morphology and had clear lipid vacuoles that could be stained with Oil Red O (Figure 7.3B; Refer to Table 2.4). Fibroblasts were similarly induced in adipocyte medium as a control. The morphology of the fibroblasts remained spindle-shaped following growth in adipocyte differentiation medium and no lipid vacuoles could be visualised using the Oil Red O stain (Figure 7.3D). Prior to induction, no lipid vacuoles were observed in either the MSCs (Figure 7.3A) or the fibroblasts (Figure 7.3C), and both cell types had a spindle-shaped morphology. The ADA patient mesenchymal stem cells differentiated in the same manner as the normal MSCs (data not shown).

7.2.2.2 Osteocyte Differentiation

Normal and patient MSCs were induced to differentiate into osteocytes for up to three weeks. The MSCs displayed in Figure 7.4 are from a normal individual, and the ADA patient MSCs differentiated in the same manner (data not shown). The non-induced and induced cells were stained with Alizarin Red S, which revealed the mineralisation of the extracellular matrix (Refer to Table 2.4). The induced MSCs stained red (Figure 7.4C), whereas the non-induced MSCs, non-induced fibroblasts and induced fibroblasts did not display any mineralisation (Figures 7.4B, D and E respectively). The non-differentiated cells retained their spindle shaped morphology (Figures 7.4B, D and E), whereas the differentiated osteocytes clearly changed

morphology and black areas of mineralisation were observed (Figures 7.4A). Osteogenesis was confirmed by measurement of calcium content in the cells. The non-induced cells and induced fibroblasts did not produce any calcium, whereas the induced MSCs produced 2mg/100mL of calcium.

7.2.2.3 Chondrocyte Differentiation

Normal and patient MSCs were induced to differentiate into chondrocytes for three weeks. Sections of 5 μM thickness were produced from the stable cartilage-like pellets of the induced cells, whereas the non-induced cells were non-viable. Murine neuroblastoma tumour sections (generous gift from Dr Susie Barker) were therefore utilised as a negative control for the chondrocyte specific staining.

To confirm differentiation, immunohistochemistry was performed on the chondrocyte and tumour sections using a chondrocyte specific antibody and stains specific for chondrocytes (Refer to Table 2.4). Chondrocyte sections from normal MSCs can be seen in Figure 7.5. Patient MSCs differentiated in a similar manner (data not shown). The sections were stained for collagen II, which is typical in articular cartilage (Figures 7.5A and B). An HRP conjugated secondary antibody revealed the presence of collagen II in the chondrocyte sections by generating a brown coloured precipitate when incubated with DAB and substrate-chromogen, not visualised in the tumour section. The chondrocyte and tumour sections were also stained with toluidine blue, which is specific for the proteoglycans within the extracellular matrix. It appears that the chondrocyte sections stain more brightly with this stain than the tumour sections, suggesting that the MSCs have differentiated into chondrocytes (Figures 7.5C and D). The sections were similarly stained using a Safranin O stain, which is specific for sulfated proteoglycans. As can be seen in Figures 7.5E and F, the chondrocytes stained more brightly red, and more of the cells stained with Safranin O than with the tumour section, suggesting chondrocyte differentiation of the MSCs. Therefore, the induced MSCs produced a stable pellet and deposited significant proteoglycan.

7.2.2.4 Neuronal Differentiation

Normal and patient MSCs were induced to differentiate into neurons for up to three weeks in the appropriate induction medium, and differences in morphology were

observed after approximately one week. The MSCs changed from a mesenchymal (7.6A) to a more typical neuronal morphology, with the cells bearing multiple processes, as can be seen in Figures 7.6B-D for normal MSCs.

Neuronal differentiated MSCs were stained with fluorescently labelled neuron specific antibodies to confirm that differentiation into neurons had taken place (Refer to Table 2.4). Although not quantified, a similar number of induced and non-induced cells were observed in the photographic frames displayed (Figure 7.7), and whereas none of the non-induced cells expressed any of the four neuronal markers tested, increased expression was observed in nearly all induced cells for three neuronal markers. Upregulation in expression was therefore observed for neuron specific enolase NSE (Figures 7.7B and C), NeuN (Figures 7.7D and E) and Tau-2 (Figures 7.7F and G). NSE is a cytoplasmic glycolytic enzyme specific to nervous tissue, NeuN stains neuronal nuclei specifically, and Tau-2 is a microtubule associated protein resulting in staining of the cell body and processes. However, no upregulation was observed with the astrocyte marker GFAP (data not shown). It therefore appears that the MSCs have differentiated into neurons.

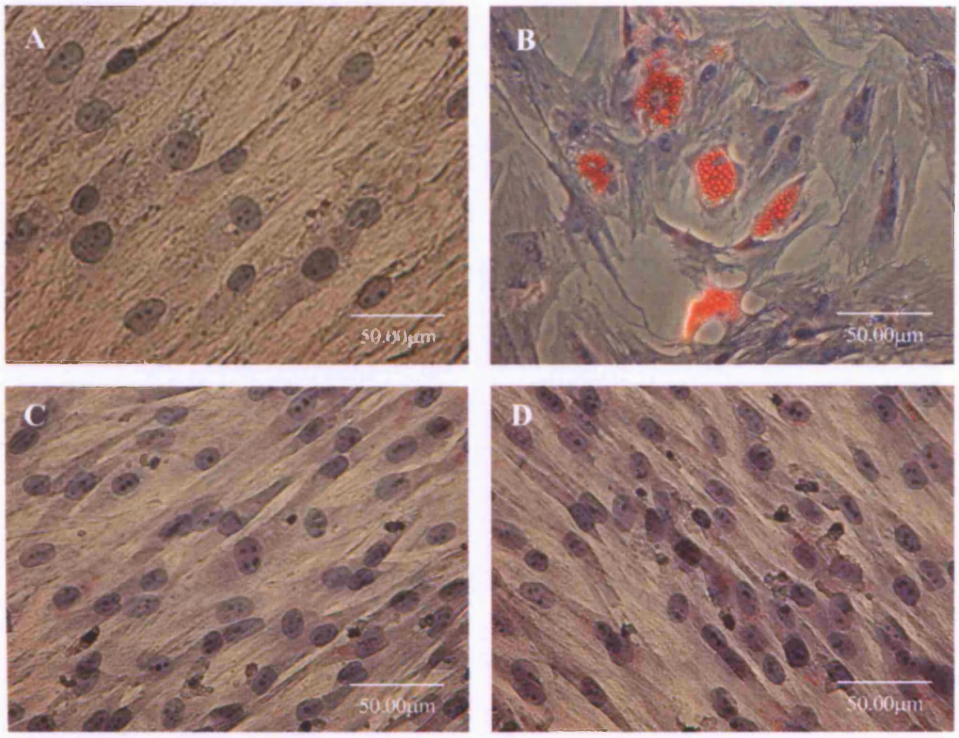
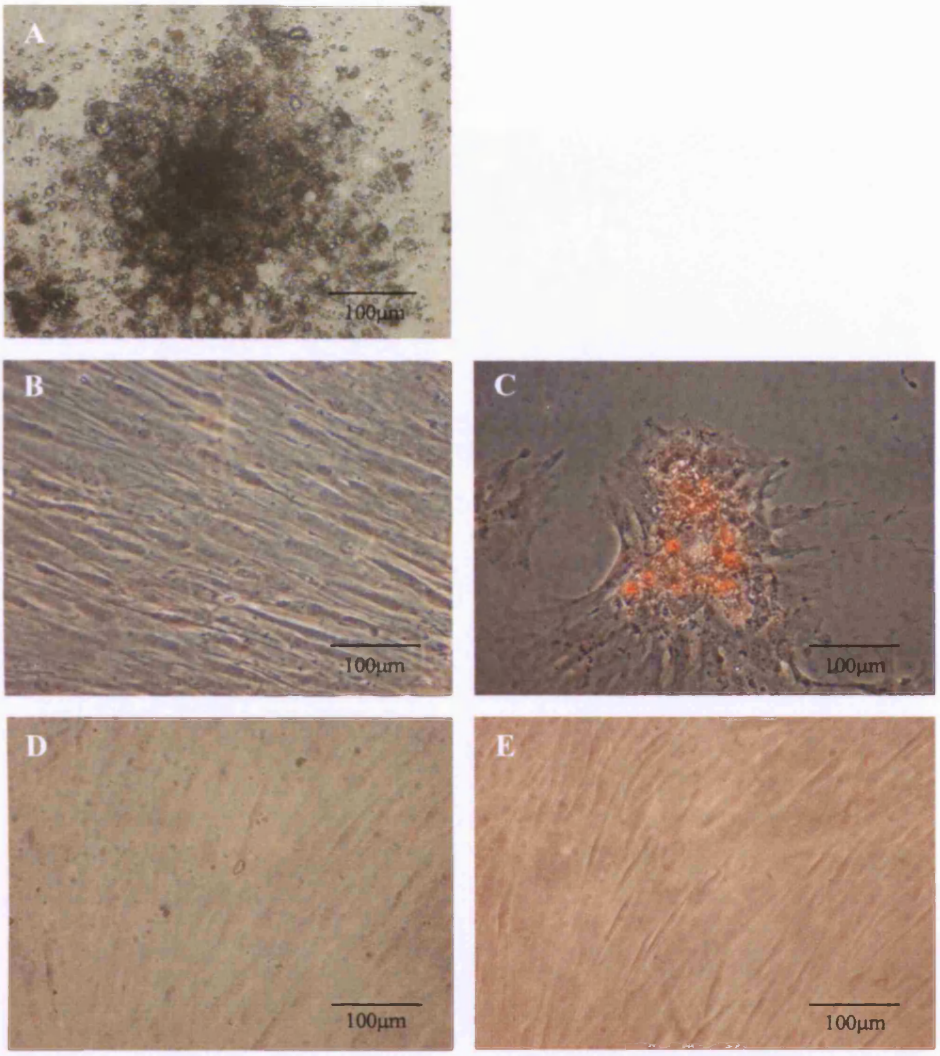


Figure 7.3. Adipocyte differentiation. Normal MSCs and fibroblasts were cultured in adipocyte inducing media and stained with Oil Red O, specific for lipid vacuoles, and haematoxylin to visualise the nucleus. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A, Non-induced MSCs; **B,** MSCs cultured in adipocyte inducing media; **C**, Non-induced fibroblasts; **D**, Fibroblasts cultured in adipocyte inducing media. Representative photographs.



Calcium deposition assay to show osteogenesis:
Non-induced MSCs – 0mg/100mL
Osteogenic induced MSCs– 2mg/100mL
Non-induced fibroblasts – 0mg/100mL
Osteogenic induced fibroblasts – 0mg/100mL

Figure 7.4. Osteocyte differentiation. Normal MSCs and fibroblasts were cultured in osteocyte inducing media and stained with Alizarin Red S to visualise mineralisation of extracellular matrix. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A, Unstained MSCs cultured in osteocyte inducing media; **B**, Non-induced MSCs stained with Alizarin Red S; **C**, MSCs cultured in osteocyte inducing media, stained with Alizarin Red S; **D**, Non-induced fibroblasts stained with Alizarin Red S; **E**, Fibroblasts cultured in osteocyte inducing media stained with Alizarin Red S. Representative photographs.

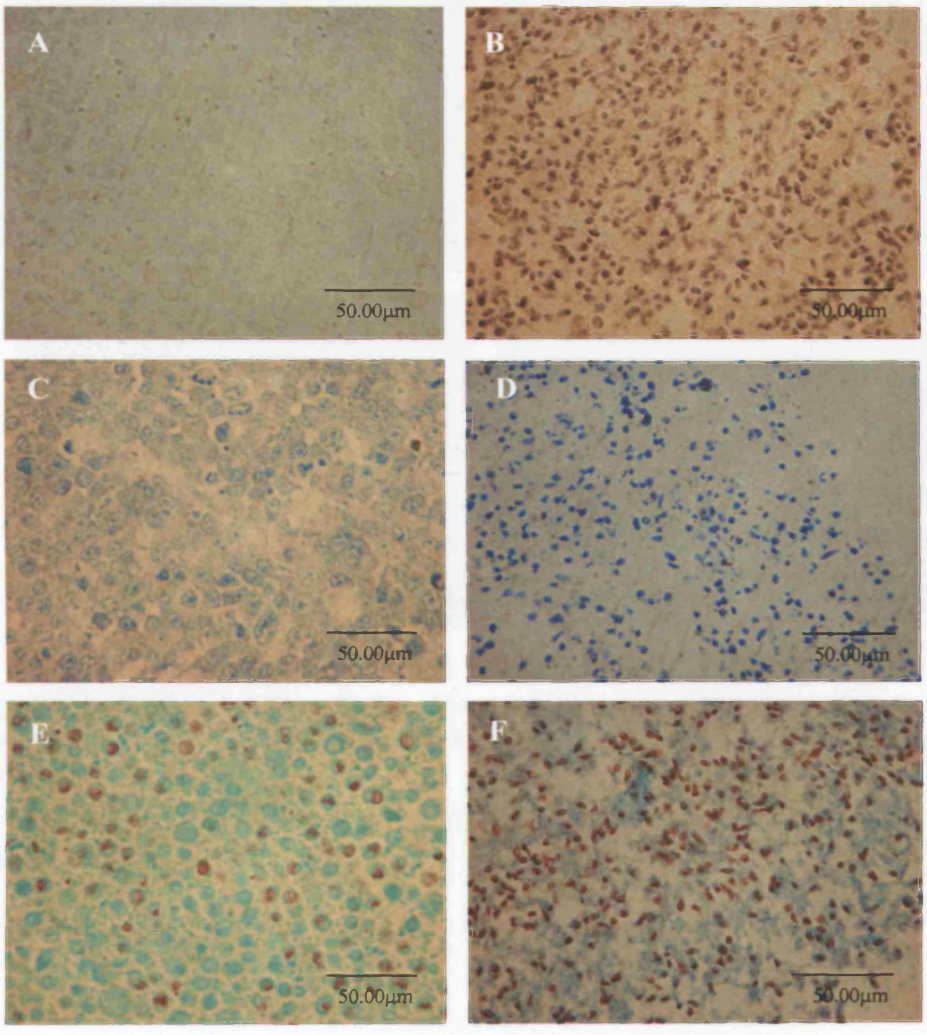


Figure 7.5. Chondrocyte differentiation. Normal mesenchymal stem cells were cultured in chondrocyte inducing medium for 3 weeks. 5 µM sections were generated and tumour sections were used as controls for the chondrocyte specific stains. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A, Tumour section stained for collagen II, typical in articular cartilage, and counterstained with methyl green. **B,** Chondrocyte section stained for collagen II and counterstained with methyl green. **C,** Tumour section stained with toluidine blue, specific for proteoglycans within the extracellular matrix. **D,** Chondrocyte section stained with toluidine blue. **E,** Tumour section stained with Safranin O, specific for sulfated proteoglycans, and counterstained with methyl green. **F,** Chondrocyte section stained with Safranin O, and counterstained with methyl green.

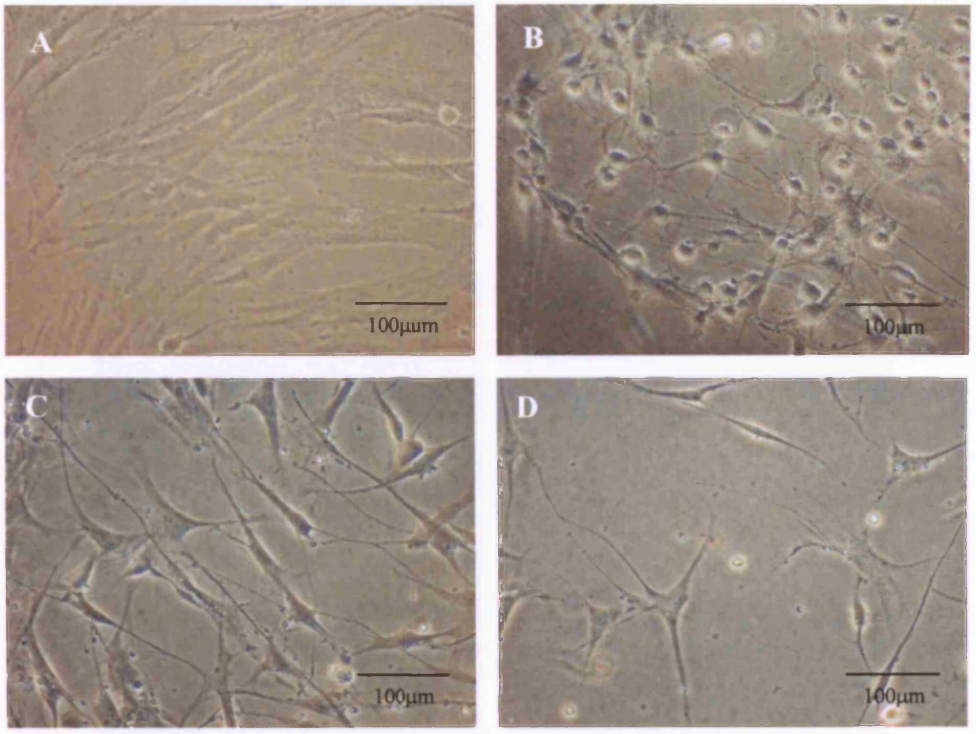


Figure 7.6. Neuronal differentiation. Normal MSCs were cultured in neural induction medium for 3 weeks. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A, Non-differentiated MSCs. **B-D,** MSCs cultured in neural induction media. Representative photographs.

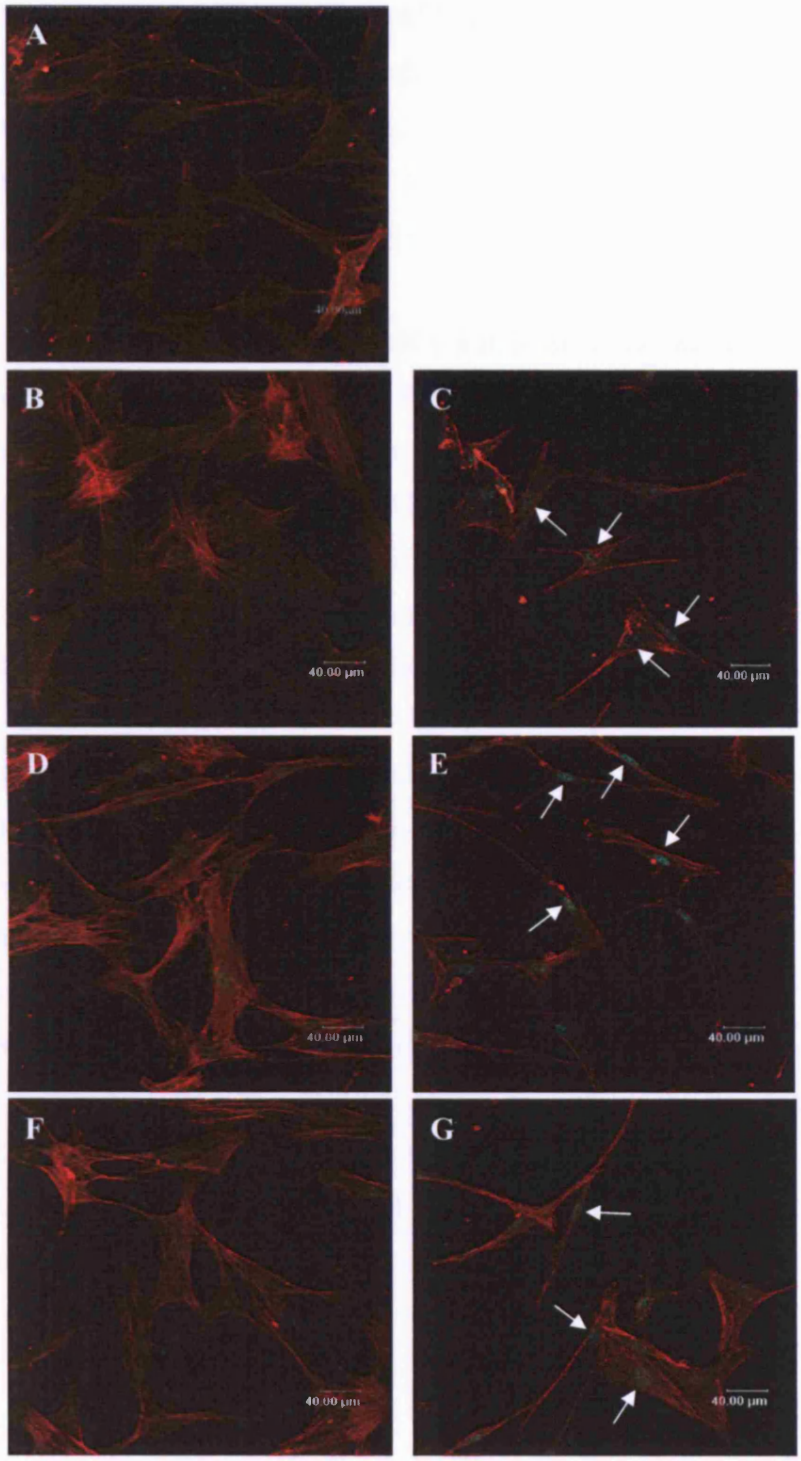


Figure 7.7. Fluorescence staining following neuronal differentiation. Patient 3 MSCs were grown in neuronal induction medium for three weeks. Confocal microscopy photographs were taken on 10-12 sections using a laser-scanning microscope (Leica, St Gallen, Switzerland). Maximal projection pictures are displayed: red – phalloidin staining for actin filaments, green – neuron specific marker as specified below. Some areas of increased fluorescence of neuronal markers have been indicated with a white arrow.

A, Secondary antibody only control; **B**, Undifferentiated MSCs stained with the neuron specific enolase (NSE); **C,** Neuronal differentiated MSCs stained with NSE; **D,** Undifferentiated MSCs stained with NeuN; **E,** Neuronal differentiated MSCs stained with NeuN; **F,** Undifferentiated MSCs stained with Tau-2; **G,** Neuronal differentiated MSCs stained with Tau-2.

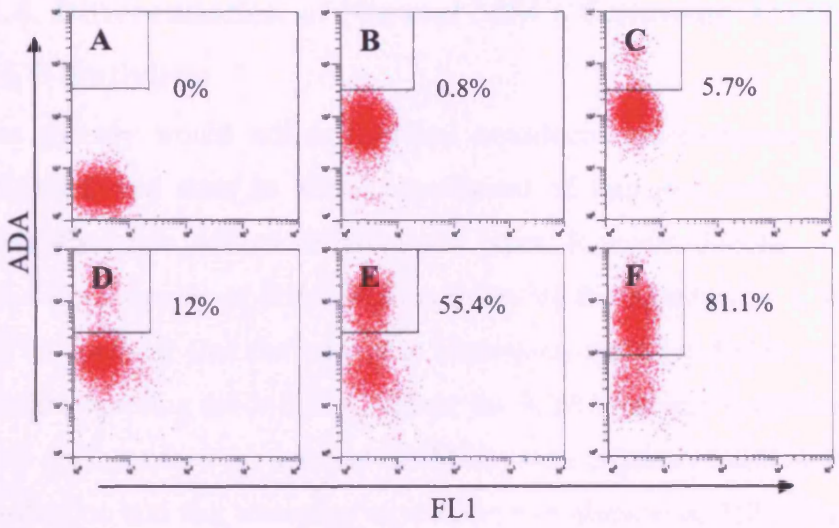
7.2.3. Transductions of Mesenchymal Stem Cells

Normal or patient MSCs were transduced with clinical grade SFada/W gammaretrovirus or with ADA-lentivirus. Three rounds of transductions were performed with the gammaretrovirus at an MOI of 2. Lentiviral transductions were performed at MOIs ranging from 1-30 using only one round of transduction.

Gammaretroviral transduction of patient MSCs was found to be inefficient; resulting in only 5.7% transduced cells at an MOI of 2 with 3 rounds of transduction (Figure 7.8C). However, flow cytometry analysis revealed that the lentivirus was more effective at gene transfer. At an MOI of 1, 12% were transduced (Figure 7.8D), MOI of 10, 55.4% were transduced (Figure 7.8E) and at an MOI of 30, 81.1% of the MSCs were transduced (Figure 7.8F). Similarly eGFP-lentivirus transduction of MSCs at an MOI of 30 resulted in 79% transduction (data not shown).

Transductions of patient MSCs resulted in large increases in ADA activity values for both gammaretroviral and lentiviral transduced cells. Normal MSCs displayed activity levels of 615.8 nmoles/hr/mg protein. Gammaretroviral transductions resulted in an increase from nearly non-existent activity levels of 20 to 6,456 nmoles/hr/mg protein, 10-fold the activity of normal MSCs. Lentiviral transductions at different MOIs gave rise to very high ADA activity values between approximately 2,000 and 200,000 nmoles/hr/mg protein, 3-fold to 300-fold higher than that of normal MSCs. Transduction with lentivirus at MOI of 1 resulted in activity levels of 2,046 nmoles/hr/mg protein; MOI of 10 resulted in activity levels of 56,880 nmoles/hr/mg protein; and MOI of 30 resulted in activity levels of 207,203 nmoles/hr/mg protein (Figures 7.8G and H).

The calculated activity levels per copy were high for all transduction conditions. The gammaretroviral transductions resulted in a slightly lower value of 165,538 nmoles/hr/mg protein per copy compared with the lentiviral transductions which ranged from 400,000 to 500,000 nmoles/hr/mg protein per copy for the different MOIs (Figure 7.8G).



G

Cells	ADA activity	% Transduction	Copy Number	Activity/Copy
Normal MSC	615.8	N/A	N/A	N/A
MH MSC	20	N/A	N/A	N/A
MH MSC + gammaretro	6,456	5.7	0.039	165,538
MH MSC + lenti MOI 1	2,046	12	0.005	409,200
MH MSC + lenti MOI 10	56,880	55.4	0.124	458,710
MH MSC + lenti MOI 30	207,203	81.1	0.414	500,490

H

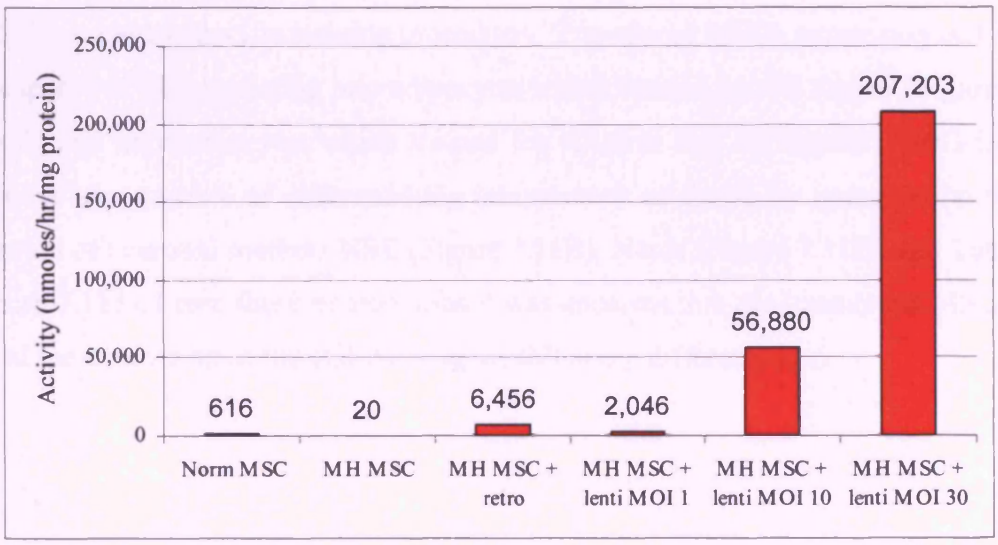


Figure 7.8. Transductions of patient mesenchymal stem cells with vectors encoding the ADA transgene. MSCs were transduced with the SFada/W gammaretroviral vector at an MOI of 2 using three rounds of transductions. MSCs were also transduced with ADA-lentivirus at MOIs ranging from 1 to 30.

Flow cytometry plots of **A,** Secondary antibody only control; **B,** Untransduced patient MSCs; **C,** Gammaretroviral transduced MSCs; **D,** Lentiviral transduced MSCs, MOI of 1; **E,** Lentiviral transduced MSCs, MOI of 10; **F,** Lentiviral transduced MSCs, MOI of 30. (The values in the flow cytometry plots refer to the ADA^+ cells). **G,** Activity values and copy numbers for untransduced and transduced MSCs. **H,** Activity values for untransduced and transduced MSCs.

7.2.4. Differentiation of Normal MSCs Following Transduction with eGFP-lentivirus

Gene therapy would utilise lentiviral transduced mesenchymal stem cells in an undifferentiated state to allow engraftment of immature cells which could then differentiate into several different cell types. It would therefore be vital that the MSCs were capable of differentiation following the transduction procedure. It would also be essential that the transgene expression remained following differentiation, therefore enabling the MSCs to deliver the ADA transgene to different sites. Hence, it was demonstrated *in vitro* that the MSCs were capable of differentiation following transduction and that transgene expression was maintained following differentiation. This was initially done by transducing normal MSCs with eGFP-lentivirus so that one could visualise the transduced, differentiated cells. Hence, MSCs were transduced with eGFP-lentivirus at an MOI of 10, which resulted in approximately 30% transduction. The flow cytometry plots displayed in Figure 7.9 confirm that even after differentiation into adipocytes, osteocytes and neurons, the eGFP expression remained in around 30-40% of the cells.

Differentiation into the different cell types was confirmed by the change in cell morphology and by specific staining procedures. Transduced MSCs expressing eGFP were capable of differentiating into adipocytes which stained for Oil Red O (Figures 7.10A-F) and into osteocytes which stained for Alizarin Red S (Figures 7.10G-L). They were also capable of differentiating into neurons as shown by upregulation of expression of neuronal markers NSE (Figure 7.11B), NeuN (Figure 7.11D) and Tau-2 (Figure 7.11F). From these photographs it was apparent that the transduced MSCs retained the expression of the eGFP transgene following differentiation.

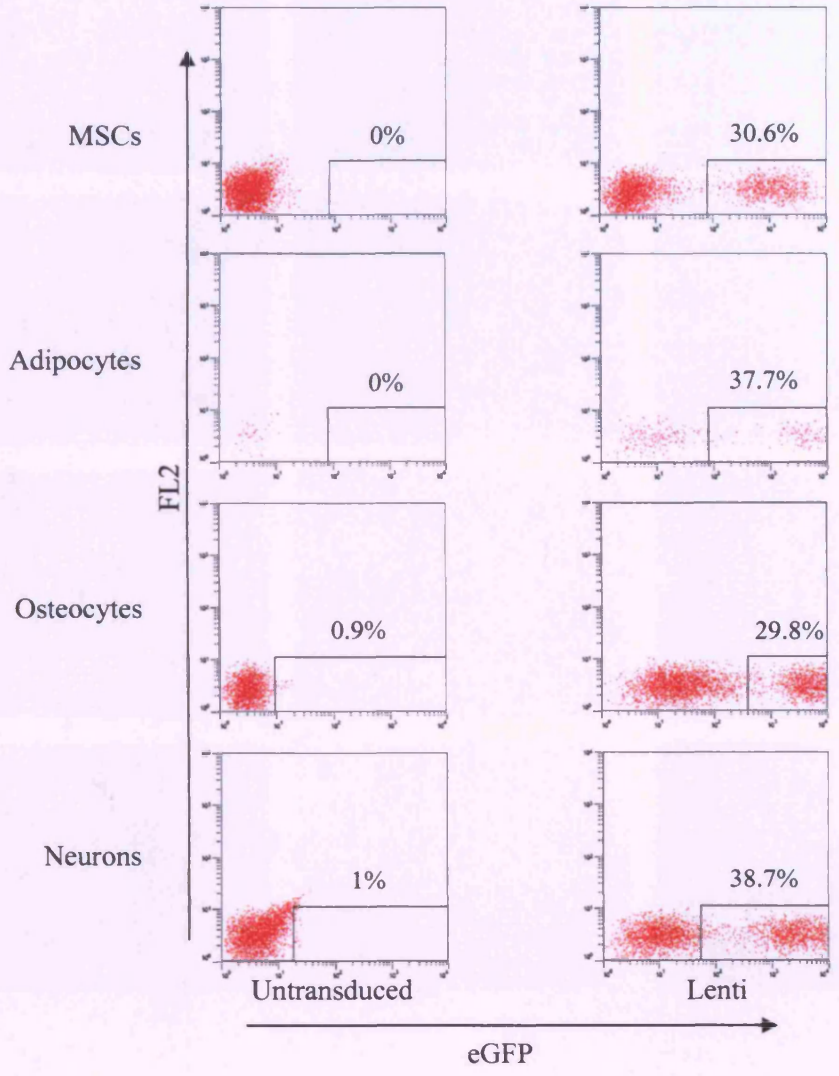


Figure 7.9. Expression of the eGFP transgene following differentiation of normal MSCs. eGFP transgene expression remains at the same level in the three differentiated cell types adipocytes, osteocytes and neurons. (The values in the flow cytometry plots refer to the GFP^+ cells).

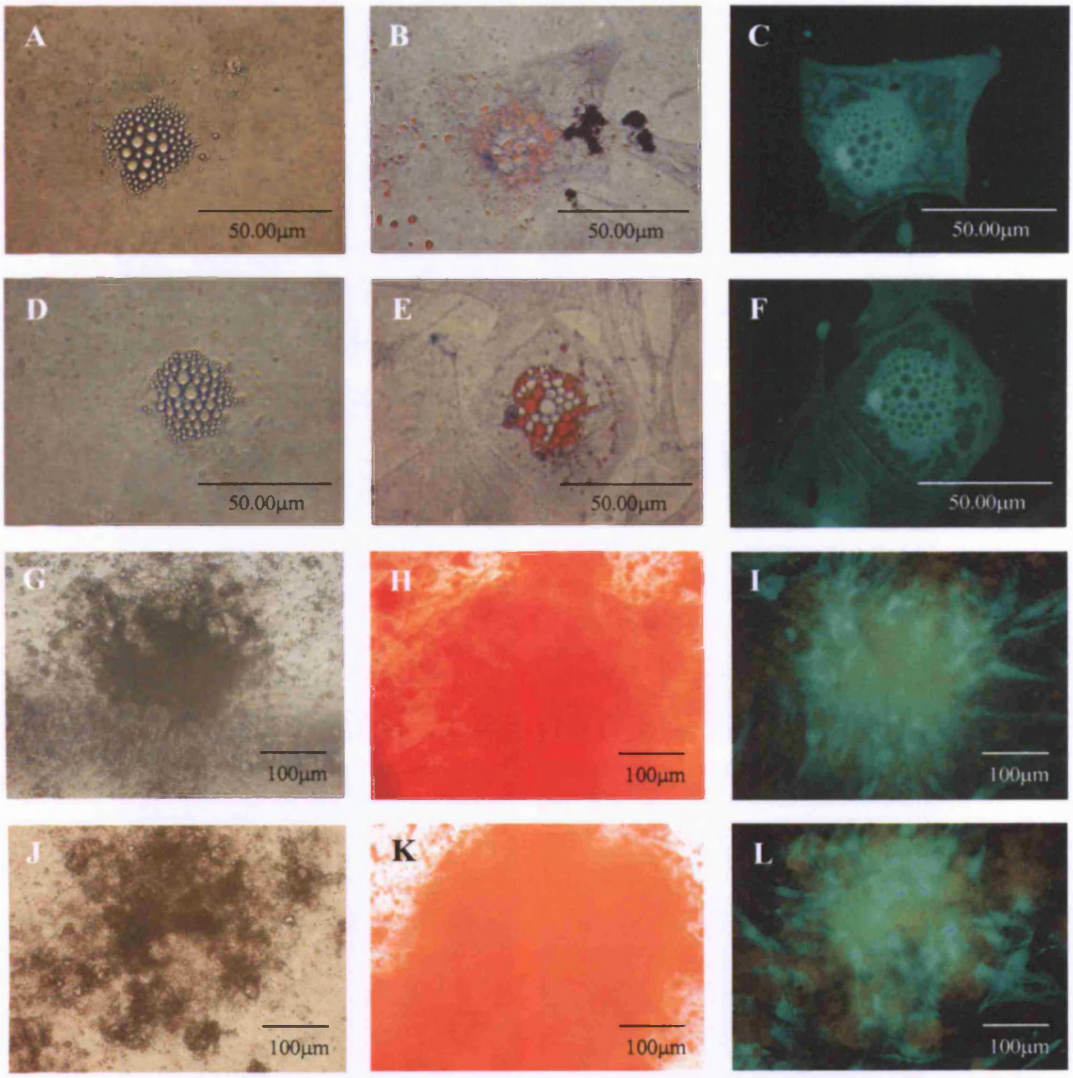


Figure 7.10. Differentiation of normal MSCs to adipocytes and osteocytes following transduction with lentivirus. The three photographs in each row displays the same cell or group of cells. Cells were visualised using the Olympus IX70 inverted fluorescence microscope.

A-F, Adipocytes expressing eGFP, showing the characteristic lipid vacuoles which stain with Oil Red O. **A-C**: same cell; **A**, unstained; **B**, stained with Oil Red O and **C**, showing eGFP expression. **D-F**: same cell; **D**, unstained; **E**, stained with Oil Red O and **F**, showing eGFP expression.

G-L, Osteocytes expressing eGFP, showing black areas of mineralisation which stain with Alizarin Red S. **G-I**: same group of cells; **G**, unstained; **H**, stained with Alizarin Red S and **I**, showing eGFP expression. **J-L**: same group of cells; **J**, unstained; **K**, stained with Alizarin Red S and **L**, showing eGFP expression.

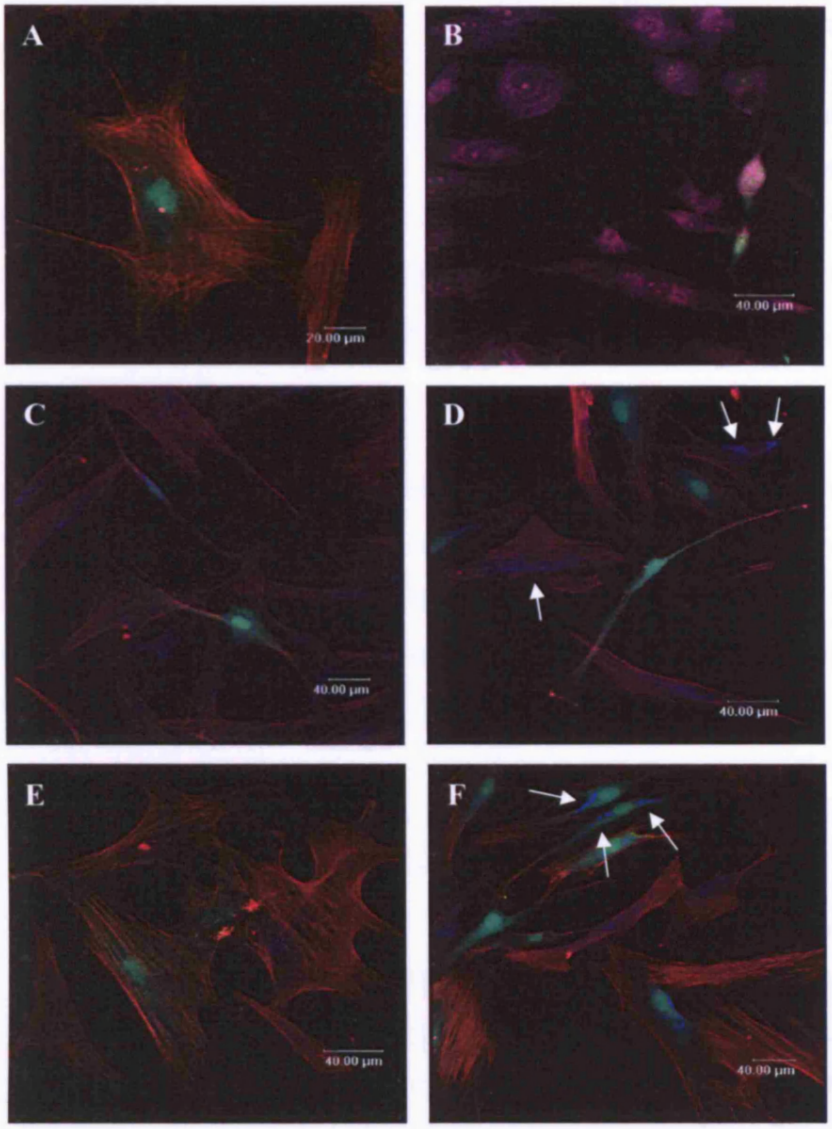


Figure 7.11. Differentiation of normal MSCs to neuronal cells following transduction with lentivirus. Confocal microscopy, maximal projection, overlay photographs taken using a laser-scanning microscope (Leica, St Gallen, Switzerland). Red – phalloidin, green – eGFP, blue – neuron specific markers as specified below. Some areas of increased fluorescence of neuronal markers have been indicated with a white arrow.

A, MSCs stained for NSE; **B,** Neuronal differentiated MSCs stained for NSE; **C,** MSCs stained for NeuN; **D,** Neuronal differentiated MSCs stained for NeuN; **E,** MSCs stained for Tau-2; **F,** Neuronal differentiated MSCs stained for Tau-2.

7.2.5. Differentiation of Patient MSCs Following Transduction with ADA-lentivirus

An alternative way of demonstrating that transduced MSCs were capable of differentiation and that differentiated cells retained the transgene expression was to generate transduced single cell clones of MSCs expressing ADA. Following transduction, the cells were sorted into single cells and expanded, after which the MSCs were of a relatively high passage of 12. These cells were then used for differentiation experiments.

A phenotype panel, similar to the one shown in Figure 7.2, was performed for two single cell clones and one untransduced patient MSC population of the same passage (Figure 7.12) to show that transduction and cell sorting does not affect the MSC phenotype. The expression pattern of the markers was mainly as expected. The MSCs were importantly negative for CD34 and CD45 and positive for SH2 (CD105) and SH4 (CD73). There are some slight differences in marker expression compared to the lower passage MSCs shown in Figure 7.2. This is likely to be due to the increase in passage rather than the transduction and sorting procedure as the high passage untransduced MSCs displayed the same markers as the high passage SCCs. The differences were as follows: a small proportion of the high passage cells expressed low levels of CD90 and a large proportion of the high passage cells have lost some of the CD106 expression. Therefore, the high passage untransduced and transduced MSCs were negative for the following markers: CD14, CD33, CD34, CD45, and CD106 (although a small proportion was still positive). They were positive for the following markers: $CD13^{high}$, $CD29^{high}$, $CD44^{high}$, CD73 (SH4), CD90 (although a proportion of the cells were negative) and CD105 (SH2) (Refer to Table 7.1).

Following confirmation of the expression of ADA in the transduced single cell clones, the cells were tested for their differentiation potential. The single cell clones were differentiated into adipocytes, osteocytes and neurons. Following adipogenic induction, the cells had a more flattened appearance and displayed the lipid vacuoles typical for adipocytes, which stained with Oil Red O (Figure 7.13B). Following osteogenic induction, the cells displayed a mineralisation of the ECM which stained

with Alizarin Red S (Figure 7.13D). The cells also differentiated into neurons. Following neuronal induction, the cells were stained for the three different neuronal markers NSE, NeuN and Tau-2. In comparison to the untransduced, undifferentiated MSCs there appears to be an upregulation in all three markers (Figures 7.13F, H and J). Although not quantified, a similar number of induced and non-induced cells were observed in the photographic frames displayed (Figure 7.13), and whereas none of the non-induced cells expressed any neuronal markers, increased expression was observed in nearly all induced cells for these three neuronal markers. These cells were also stained for the astrocyte marker GFAP, which showed no upregulation in expression (data not shown). It therefore appears that the MSCs, following transduction, are capable of differentiating into neurons. Hence, in comparison to untransduced MSCs (Figures 7.3-7.7) the transduced cells differentiate in a similar manner, as assessed by morphology and by specific staining procedures.

Real-time PCR analysis was performed on the MSC single cells clones (SCCs) to determine the copy number of the ADA transgene. It was found that both of the SCCs had a calculated copy number of approximately 0.3-0.5 (Figure 7.14A), which is likely to mean that the cells carry one copy of the ADA transgene.

Flow cytometry analysis of the different MSC populations revealed that ADA transgene expression persisted following differentiation into adipocytes, osteocytes and neurons (Figure 7.14B). Hence, differentiation does not affect transgene expression levels.

Therefore, although the transduced MSCs have a slightly changed marker phenotype, probably due to the high passage of the cells, they are still capable of differentiation into three different lineages. Importantly, following these differentiations, the cells retained their ADA expression.

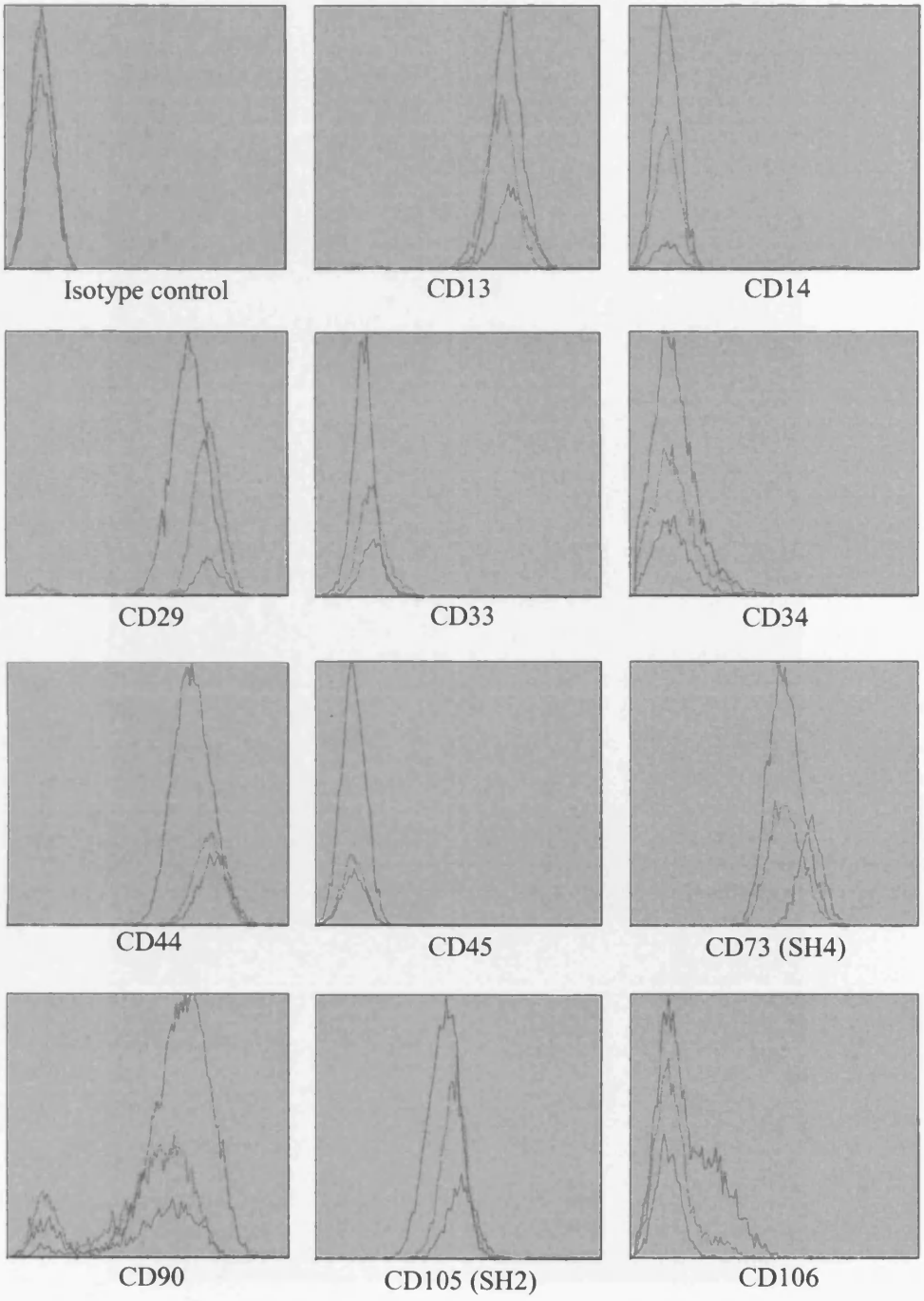


Figure 7.12. Patient MSC single cell clone phenotype panel. Flow cytometry plots displaying the phenotype panel of two transduced single cell clones in comparison to untransduced MSCs. Red – untransduced MSCs, pink – SCC1, green –SCC2.

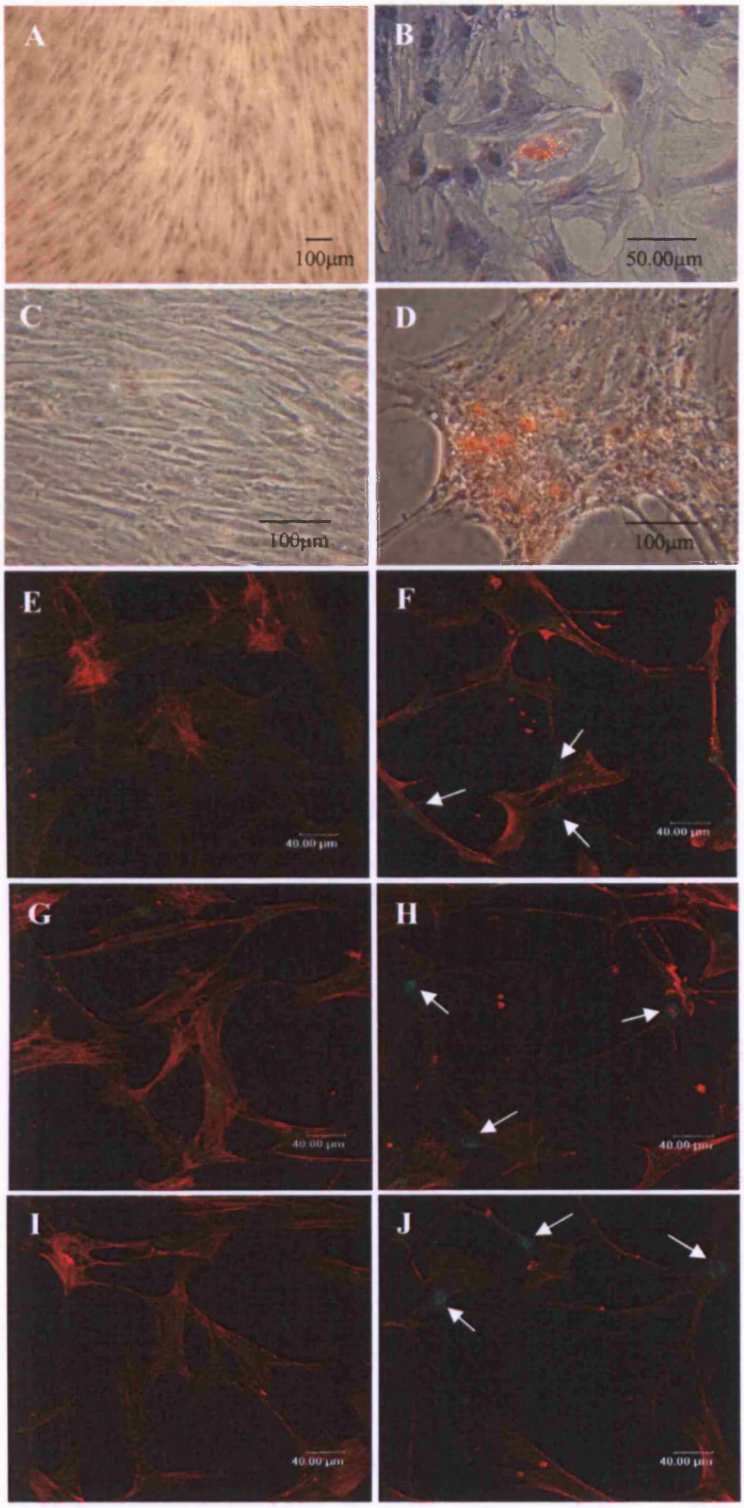


Figure 7.13. Differentiation of transduced patient MSC SCCs. Following ADA-lentiviral transduction, the MSCs were differentiated into adipocytes, osteocytes and neurons. Some areas of increased fluorescence of neuronal markers have been indicated with a white arrow.

A-D, Cells were visualised using the Olympus IX70 inverted fluorescence microscope. **A,** MSCs stained with Oil Red O; **B**, Adipocyte differentiated MSCs stained with Oil Red O; **C**, MSCs stained with Alizarin Red S; **D**, Osteocyte differentiated MSCs stained with Alizarin Red S; **E-J** Confocal microscopy maximal projection overlay photographs taken using a laser-scanning microscope (Leica, St Gallen, Switzerland) , red – phalloidin, green – neuron specific marker. Undifferentiated cells are untransduced. **E,** MSCs stained for NSE; **F,** Neuronal differentiated MSCs stained for NSE; **G,** MSCs stained for NeuN; **H,** Neuronal differentiated MSCs stained for NeuN; **I,** MSCs stained for Tau-2; **J,** Neuronal differentiated MSCs stained for Tau-2. Mag, magnification.

A

Copy Numbers	MSCs	Adipocytes	Osteocytes	Neural
Untransduced	0	0	0	0
Lenti SCC1	0.493	0.364	0.386	0.410
Lenti SCC2	0.309	0.327	0.421	0.502

B

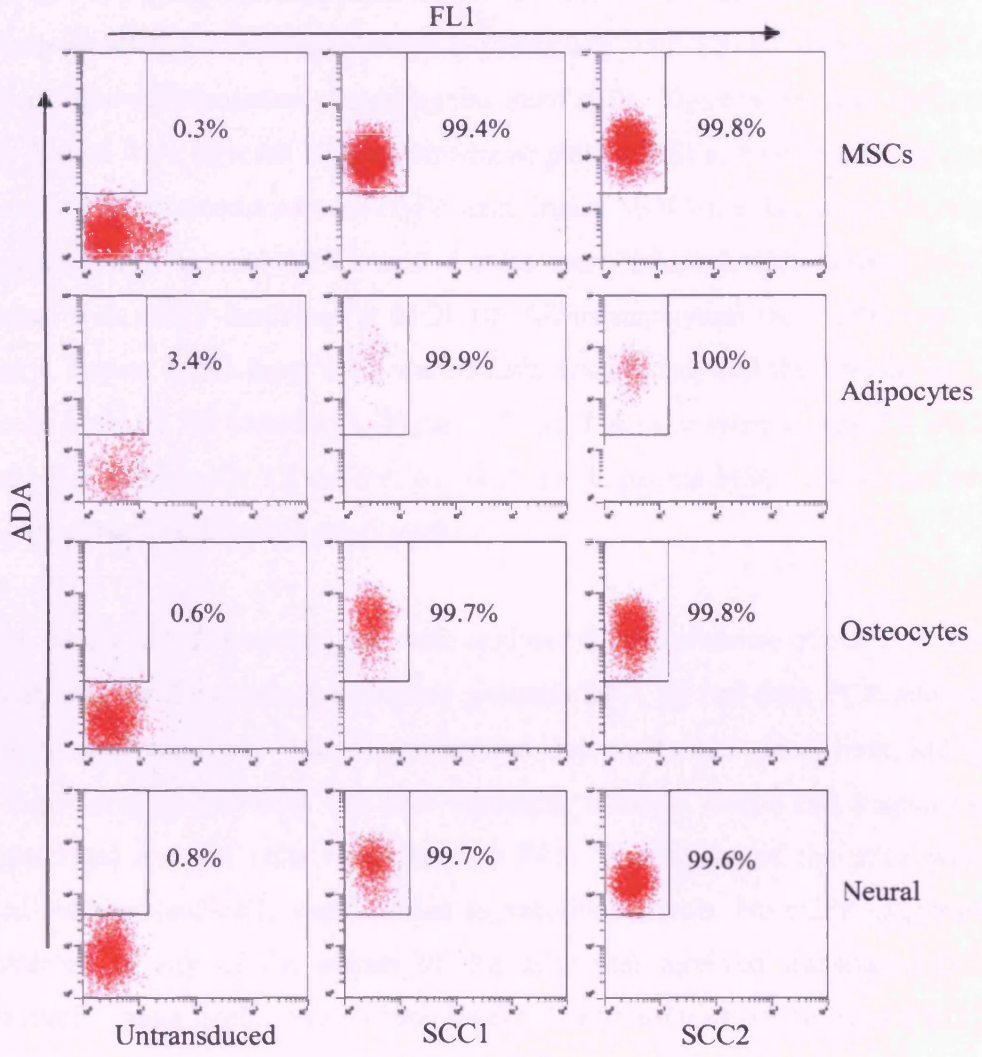


Figure 7.14. Retention of ADA transgene presence and expression following differentiation of patient MSCs.

A, Copy numbers of ADA transgene in the two single cell clones prior to and following differentiation. **B,** Flow cytometry plots displaying the expression of ADA in untransduced and 2 single cell clones prior to and following differentiation into 3 different cell types.

7.2.6. Homing and Engraftment of MSCs in NOD-SCID Mice

It was important to assess the homing and engraftment abilities of MSCs, as ADA needs to be delivered to several different organs in ADA deficient patients. Prior to initiation of gene therapy trials it will be necessary to demonstrate in an animal model that MSCs are capable of homing to the organs of interest, including the liver and the brain, and engrafting in those organs. Hence, in order to evaluate the homing abilities of MSCs, the xenotransplant model the NOD-SCID mouse was utilised. 13 sublethally irradiated mice were injected intravenously with $0.5x10^6$ untransduced or transduced non-differentiated mesenchymal stem cells. There were four different groups; 2 mice were injected with untransduced patient MSCs, 6 mice were injected with patient MSCs transduced with eGFP-lentivirus at MOI 10, 1 mouse was injected with untransduced normal MSCs and 4 mice were injected with normal MSCs transduced with eGFP-lentivirus at MOI 10. All mesenchymal stem cells were of passage 9. Patient MSC input cells were 57.6% transduced, and the normal MSCs input cells were 67.7% transduced (Figure 7.15A). The mice were analysed 6 weeks after injection of the cells. Of the 6 mice injected with patient MSCs transduced with eGFP-lentivirus, 3 died before the 6 weeks.

For each mouse, ten different organs were analysed for the presence of eGFP by flow cytometry and for the presence of human genomic DNA by real-time PCR analysis. The organs analysed were: blood, bone marrow, spleen, thyroid gland, liver, kidney, brain, heart, lung and muscle. For flow cytometry analysis, single cell suspensions were generated and the cells were fixed in PFA. The organs of the mice which received untransduced cells were utilised as negative controls. No eGFP expression was detected in any of the organs of the mice that received transduced cells. Representative plots from 2 mice which received untransduced or transduced MSCs are displayed in Figure 7.15B.

A small section of tissue from each organ was used to isolate genomic DNA. Real-time PCR analysis was performed on the DNA generated, using primers for human β-actin. This is a very sensitive assay, detecting as few as 10 cells in a sample, which should reveal the presence of human cells within the mouse organs, irrespective of the presence of eGFP. Each of the ten organs of the ten mice were analysed for the

presence of β-actin, however no human DNA was detectable (Figure 7.16A). A positive control of human fibroblasts was run in parallel to ensure that the assay was functioning, and this gave a clear positive signal (Figure 7.16A). To verify that there was human or mouse genomic DNA present of good amplifiable quality, a standard PCR was performed using primers for HPRT specific for either human or mouse DNA (representative samples are shown in Figure 7.16B). This revealed that there was good quality DNA present and hence confirmed that the result obtained for β-actin was negative.

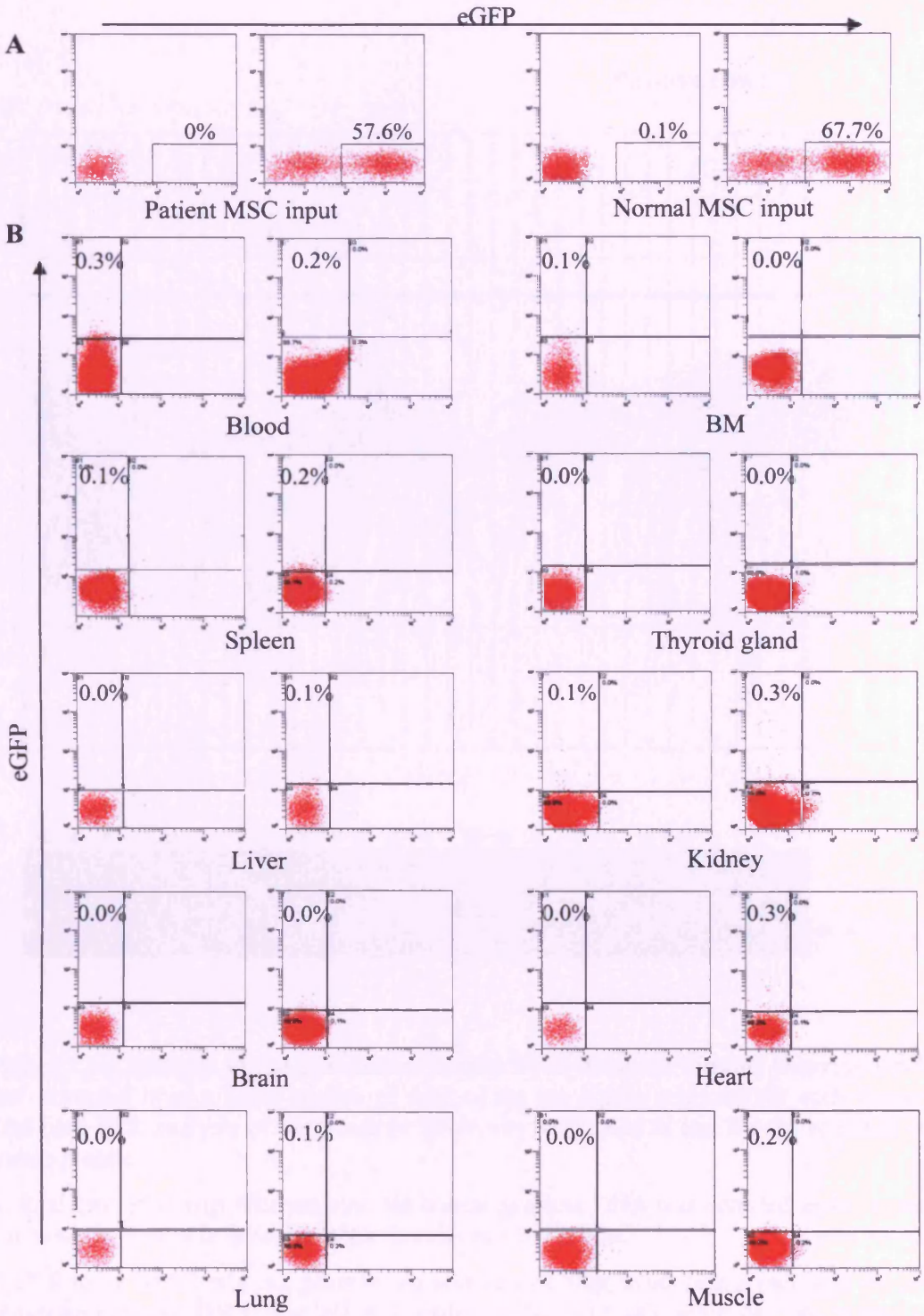


Figure 7.15. Analysis of eGFP expression in cells of different mouse organs. Lentivirus-eGFP transduced MSCs were injected into NOD-SCID mice. 6 weeks following injection, 10 different organs from each mouse were analysed for the presence of the eGFP transgene by flow cytometry. Samples have been gated on the forward scatter/side scatter characteristics to select cells of each organ type and to exclude dead cells and debris.

A, eGFP expression of MSC input cells (left-hand side plots are untransduced cells and right-hand side plots are transduced cells).

B, Flow cytometry plots displaying eGFP expression of cells of different organs. For each organ, the left hand side plots depicts the organ from a mouse which received untransduced normal MSCs and the right hand side plot depicts the organ from a mouse which received transduced normal MSCs. (The values in the flow cytometry plots refer to $eGFP^+$ cells)

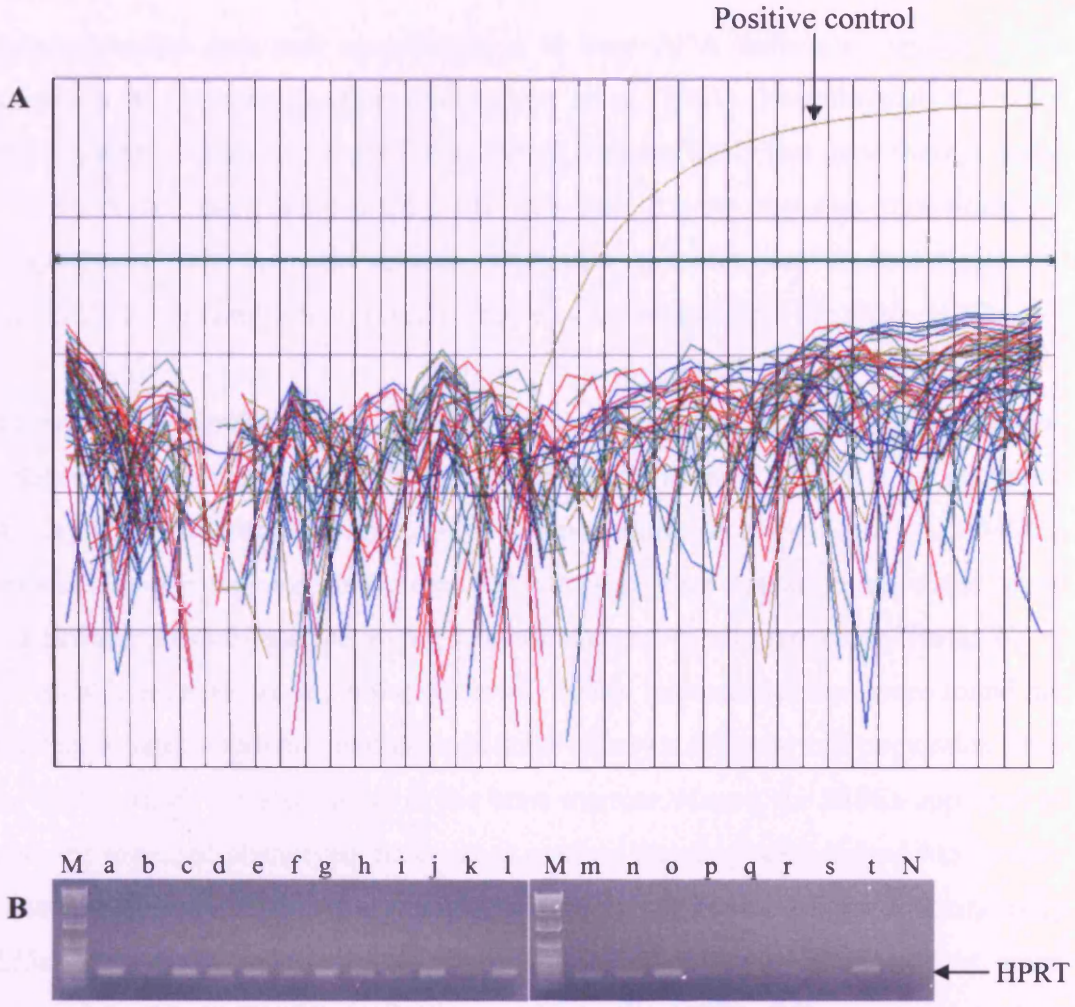


Figure 7.16. Analysis of the presence of human DNA in mouse organs. Genomic DNA was extracted from a tissue section of each of the ten organs analysed for each mouse. Real-time PCR analysis of the genomic DNA was performed to test for the presence of human β-actin.

A, Real time PCR amplification plot. No human genomic DNA was detected in 40 cycles. The positive control is genomic DNA from human fibroblasts.

B, PCR using HPRT primers, generating a product of 220bp, to confirm presence of human or mouse genomic DNA. The gel was loaded in the following order: M, 1kb ladder; a, Blood 1; b, bone marrow 1; c, spleen 2; d, thyroid gland 2; e, liver 3; f, kidney 3; g, brain 4; h, heart 4; i, lung 5; j, muscle 5; k, blood 6; l, bone marrow 6; M, 1kb ladder; m, spleen 7; n, thyroid gland 7; o, liver 8; p, kidney 8; q, brain 9; r, heart 9; s, lung 10; t, muscle 10; N, negative control (numbers refer to different mice).

7.3. DISCUSSION

Haematopoietic stem cell transplantation to treat ADA deficiency results in the correction of immune function (Hirschhorn *et al.*, 1981). Neurological and other organ system problems appear to remain, and it is also likely that gene therapy using T cells or haematopoietic stem cells will fail to treat the non-immunological symptoms of this systemic disease. Therefore, the aim was to investigate the possibility of utilising MSCs to offer a more systemic treatment for ADA-SCID.

Homogeneous populations of mesenchymal stem cells were readily generated by the isolation of mononuclear cells from bone marrow, followed by plastic adherence of MSCs. To verify the homogeneity of the population and the identity as MSCs, immunophenotyping was performed. The MSCs were found to have the same expression pattern of surface markers as has been previously reported (Zhang, W., *et al.*, 2004; Lee, R.H., *et al.*, 2004; Xu *et al.*, 2004). Importantly, they were found not to express haematopoietic markers, and were clearly a different cell population from the HSCs which are also found in the bone marrow. Hence, the MSCs appeared to have the expected phenotype, however to confirm that they were indeed MSCs their differentiation potential was assessed. Importantly, the MSCs did not spontaneously differentiate during culture unless grown in differentiation medium. However, when cultured in the appropriate induction medium, the MSCs differentiated into four different cell types (adipocytes, osteocytes, chondrocytes and neuronal cells) assessed by their change in morphology and by specific staining assays. Increased expression was seen for the three neuronal markers NSE, NeuN and Tau-2, whereas no upregulation was observed with the astrocyte marker GFAP, suggesting that the MSCs have differentiated into neurons and not astrocytes by this differentiation method using hEGF and hbFGF. The expression pattern for each of the three markers appears to be nuclear or peri-nuclear. Some faint cytoplasmic staining can also be observed for NeuN and Tau-2. NSE is a cytoplasmic glycolytic enzyme specific to nervous tissue, NeuN is a neuronal nuclei protein and Tau-2 is a microtubule associated protein. Therefore, NSE and Tau-2 staining would be expected to be mainly cytoplasmic and NeuN staining should be mainly nuclear in brain derived neurons. However, recent reports (Lu *et al.*, 2004) have revealed that visualisation of neuronal markers, as performed in this study, may not be adequate to confirm the

identity of the cells. They showed that the "induction of neuronal differentiation" by addition of specific chemicals resulted in cellular shrinkage and thus increased the concentration or apparent upregulation of neuronal markers, although not differentiation. Moreover, it has been shown that mesenchymal derived neurons may have different distributions of these neuronal markers (Jin *et al.*, 2003) compared with brain derived neurons, which may explain the slight difference in cellular distribution observed for these markers in the present study. This may indicate that the mesenchymal derived neurons do not have the functional characteristics of neurons. Therefore, in order to conclusively prove that the MSCs have differentiated into neuronal cells, one would not only need to demonstrate the expression of neuronal markers but also that the cells have acquired the electrophysiological properties of neurons. Hence, measurement of the action potential and voltage gated Na^+ and K^+ currents would be required as has been previously demonstrated (Li *et al.*, 2005; Cho *et al.*, 2005).

Once the MSCs had been shown to express the characteristic markers and to differentiate into four different cell types, viral transductions were performed. Gammaretroviral transductions were found to be greatly inefficient, which may be due to a lack of Pit-1 GaLV receptors on the surface of the MSCs or due to their low rate of cell division. In a homogeneous population of MSCs it has been found that most of the cells are standing at the G_0/G_1 phase of the cell cycle (Conget & Minguell, 1999), which could explain the difficulties in transducing MSCs using a gammaretrovirus that can only transduce dividing cells. However, other groups have obtained transduction efficiencies of approximately 80% using different protocols such as high titre gammaretroviruses pseudotyped with VSV-G or amphotropic envelope (Marx *et al.*, 1999; Baxter *et al.*, 2002; Mangi *et al.*, 2003) or centrifugation of cells together with the gammaretrovirus (Lee, K., *et al.*, 2001). These high transduction efficiencies could not be obtained in the present study, and the differences in transduction procedure, the centrifugation step, the high titre and different envelopes of the gammaretroviruses, may explain the differences in transduction efficiencies obtained. Perhaps the MSCs express higher levels of Pit-2 (amphotropic receptor) or VSV-G receptors than Pit-1, which the viral GaLV envelope targets. The expression of these receptors on MSCs should be further investigated if the gammaretrovirus was the vector of choice. However, whereas

consistently low transduction efficiencies were observed with gammaretroviral vectors, lentiviral transductions were extremely efficient. Reasons as to why lentiviral transductions were much more efficient than gammaretroviral transductions may be due to the cPPT element that permits the lentivirus to transduce these quiescent cells which are mainly in the G_0/G_1 stage of the cell cycle, or due to the different viral envelopes. Previous studies using lentiviral vectors have obtained slightly higher gene transfer efficiencies than observed here, with transductions at an MOI of 1 resulting in approximately 50% transduced cells (Zhang *et al.*, 2002; Totsugawa *et al.*, 2002). Similarly to the fibroblasts and LCLs, the transduced MSCs in our study were found to maintain the expression of the transgene for a long period of over 14 passages (more than 4 months). This is important as sustained expression of the ADA transgene would be essential for the treatment of ADA deficiency.

Interestingly, the copy numbers and activity values obtained for the lentiviral transductions of the MSCs are quite different from the fibroblasts although the percentage gene transfer was similar at an MOI of 30. Lentiviral transductions of fibroblasts resulted in a high average copy number of 8, whereas the same transductions only resulted in an average copy number of 0.4 in the MSCs. This may suggest that all of the transduced MSCs have only one copy of the transgene, whereas each transduced fibroblast has several copies. This may be related to the fact that fibroblasts were more easily transduced. The activity values of transduced MSCs are nearly twenty times lower than the transduced fibroblasts. However, this is likely to be as a result of the difference in copy number, as the calculated values of activity per copy are nearly identical for the two cell types at around 500,000 nmoles/hr/mg protein per copy. Therefore, as expected, each copy of the lentiviral transgene gives rise to a similar activity value.

Nevertheless, the activity values observed for the transduced MSCs should be more than adequate for gene therapy. Values ranging from 2,000 to 200,000 nmoles/hr/mg protein were obtained for the different MOIs, which is 3-300 times higher than the ADA activity in normal MSCs. These activity values should clearly be sufficient to detoxify the MSCs as even the cells transduced at a low MOI display an ADA activity higher than normal.

It was important to demonstrate that the MSCs were capable of differentiation following transduction. The maintenance of MSC phenotype and function as well as transgene expression following differentiation was demonstrated by generating single cell clones which expressed ADA. The phenotype of transduced single cell clones was shown to be similar to untransduced low passage MSCs apart from the down-regulation of the marker CD106, and some loss in the expression of CD90. However, this was probably due to the high passage of the cells rather than the transduction procedure as high passage untransduced cells showed the same phenotype. Similar down-regulations in marker expressions have also been observed in other studies. Conget and Minguell (1999) found that MSCs of high passage lost expression of SH3, ICAM and integrin β1. The transduced single cell clones were importantly negative for haematopoietic markers and positive for SH2 and SH3, hence the transduction procedure did not affect the MSC phenotype.

It was also vital to demonstrate that transduced cells were capable of differentiation, and that differentiation did not affect transgene expression. Transduced MSCs were found to differentiate into the three different lineages tested: adipocytes, osteocytes and neurons. The expression of the ADA or eGFP transgene remained following differentiation. Hence, transduced MSCs remained multipotential and transgene expression was not affected by differentiation. Similar studies have demonstrated the maintained differentiation potential following gammaretroviral transduction (Lee, K., *et al.*, 2001; Baxter *et al.*, 2002) and lentiviral transductions (Quintavalla *et al.*, 2002; Zhang, X.Y., *et al.*, 2002), and the sustained transgene expression following differentiation.

Following the generation of multipotential MSCs, we investigated the homing abilities of these cells to observe into which organs they engraft. MSCs were tagged with an eGFP marker using the lentiviral vector and were then injected into NOD-SCID mice. Of a group of 6 mice injected with transduced patient MSCs, 3 mice died. As they were all from the same group, there may have been an infection in the cell culture that the immunodeficient mice could not fight. Because the NOD-SCID mice do not have a completely functional immune system, the human MSCs should not be rejected. However, no eGFP positive cells and no human cells could be detected in any of the ten different organs of the ten mice analysed. The absence of

eGFP expression in the organs analysed is unlikely to be due to gene silencing, as real-time PCR analysis did not detect the presence of human cells using β-actin markers. It therefore appears that the MSCs did not engraft in the mice, contrary to findings of some previous reports. A successful engraftment study was carried out by Pereira and colleagues (Pereira *et al.*, 1995; reviewed by Prockop, 1997) who infused marked MSCs into X-ray irradiated mice, and analysed several tissues at different time points. After 1 week, no MSCs could be detected in any tissues. However, at 1 and 5 months the MSCs accounted for 1 to 12% of the cells in a number of tissues including bone, cartilage, lung, marrow and spleen. Other groups have similarly reported engraftments in mice (Dao *et al.*, 1997; Allay *et al.*, 1997; Chuah *et al.*, 2000) and different animal models (Devine *et al.*, 2001; Saito *et al.*, 2002; reviewed by Krause, 2002). However, our negative results could be due to a number of reasons. It is possible that too few cells were injected as $5x10^5$ cells may not be enough to be able to detect after 6 weeks, considering that only a small percentage of cells usually engraft. It is also conceivable that the cells were of too high a passage, and perhaps cells of a passage lower than 9 would have engrafted. This is supported by studies carried out by Baxter *et al.* (2004) who reported that even minimal expansions of MSCs induce rapid aging of the cells, thus probably reducing their long-term engraftment potential *in vivo*. Rombouts & Ploemacher (Rombouts & Ploemacher, 2003) also found that culturing of MSC significantly reduced their homing abilities. It that study it was observed that in contrast to transplantations of primary uncultured MSC which gave rise to 55-65% recovered cells in BM (24 hours post-transplant), following 24 hours in culture the recovery was dramatically decreased and following only 48 hours in culture, no MSCs could be detected in the lymphohaematopoietic organs. Protocols for engraftment of MSCs may therefore need to be optimised, as other groups have similarly found MSC engraftment difficult to achieve (Friedenstein *et al.*, 1978; Laver *et al.*, 1987; Simmons *et al.*, 1987; Athanasou *et al.*, 1990; Santucci *et al.*, 1992; Tanaka *et al.*, 1994). Hence, to assess the homing abilities of MSCs, the experiment should be repeated using a higher number of lower passage MSCs, perhaps injected with irradiated carrier cells to encourage engraftment of the MSCs.

Therefore, MSCs were readily generated by the plastic adherence method and were multipotential as demonstrated by their ability to differentiate into adipocytes,

osteocytes, chondrocytes and neurons. Although the homing abilities of the MSCs could not be demonstrated, it was shown that the MSCs were capable of differentiation prior to and following transduction and that the expression of the transgene remained following expansion and differentiation. As ADA deficient patients have good numbers of both HSCs and MSCs in the bone marrow, gene therapy using lentiviral vectors to transduce these cells would be feasible. If engraftment is obtained with the MSCs, the levels of ADA activity observed with the lentiviral transductions should be sufficient to reduce toxicity of the ADA substrates. Moreover, engraftment of HSCs in combination with MSCs may improve the engraftment levels of HSCs as MSCs have been found to provide support for haematopoiesis. Therefore gene therapy using lentiviral vectors to transduce HSCs and MSCs could constitute a successful systemic treatment for ADA deficiency.

8

GENERAL DISCUSSION

8.1 PATIENT IMMUNE FUNCTION FOLLOWING PEG-ADA OR GENE THERAPY

Three ADA deficient patients who did not have bone marrow donors available were treated with PEG-ADA enzyme replacement therapy. Patients 1 and 2, who remain on PEG-ADA, have responded well to this treatment displaying a good proportion of naïve cells and thymic activity, and lymphocyte counts just below normal. The immune function of patient 3 did not improve to the same extent as those for patients 1 and 2 as a result of PEG-ADA. Therefore, patient 3 was enrolled in a Phase I gene therapy trial using the previously tested SFada/W vector to transduce autologous $CD34^+$ cells.

Based on the recent successful treatment of ADA deficient patients (Aiuti *et al.*, 2002a), a protocol was designed for the ADA gene therapy trial. This involved the withdrawal of PEG-ADA prior to gene therapy in order to maximise the selective growth advantage of transduced cells, and a mild conditioning treatment to generate space for reinfused cells. Patient 3 was therefore treated using a similar protocol to previously published gene therapy trials. Following the infusion of transduced cells, the transgene could be detected in the patient's mononuclear cells up to sixteen months post gene therapy. The patient now appears to be generating a larger proportion of naïve cells compared to prior to gene therapy and lymphocyte counts are higher than when he was treated with PEG-ADA.

When patient 3 received PEG-ADA, he did not respond as well to this treatment as patients 1 and 2 as determined by lower lymphocyte counts and a smaller proportion of naïve cells. Following gene therapy the immune function of patient 3 improved significantly, however, his immune recovery was still not as good as for patients 1 and 2 being treated with PEG-ADA. It would therefore be interesting to follow this further to determine why patients 1 and 2 responded better to treatment than patient 3. It could for instance be due to the mutation carried by the patient, as Hirschhorn *et al.* (1993) suggested that genotype is related to phenotype and response to treatment. It was hypothesised that patients with mutations allowing for some ADA expression might respond better to PEG-ADA therapy. There are clearly several other factors

which could also contribute to the response to treatment, such as age at treatment and previous infections. It has been proposed that treatment of X-SCID patients at an older age results in limited initiation of thymopoiesis and T cell recovery (Thrasher *et al.*, 2005), and this is likely to hold true for ADA-deficient patients. Furthermore, the lack of ADA during foetal development may result in different extent of damage to the foetus. It may therefore be difficult to determine the reasons for different treatment responses in patients. Mutational analysis and evaluation of T cell functions and thymic function at an early age might clarify the differences in treatment response.

Further similar studies should therefore be carried out on ADA deficient patients treated with either bone marrow transplantation, PEG-ADA or gene therapy. It would be interesting to compare immunological recovery and improvements of non-immunological functions as a result of the different treatments. Although no study with direct comparisons of the different treatments have been done to date, there have already been some suggestions regarding the efficacies of the different treatments. The conventional treatments of bone marrow transplantations and PEG-ADA are both thought to have certain limitations. Bone marrow transplantations, for instance, are known to improve immune function, however the metabolic abnormalities have been found to remain quite high in other organs (Hirschhorn *et al.*, 1981). Therefore, improvements of non-immunological defects may be poor. Although not yet proven, it is thought that PEG-ADA, being a more systemic treatment than BMT, may be a better treatment for neurological symptoms and other non-immunological defects (Rogers *et al.*, 2001; Albuquerque *et al.*, 2004). However, the efficacy of this treatment may diminish with time, particularly if antibodies are generated against the bovine ADA. Hence, gene therapy may be an improved treatment for ADA deficient patients as it results in a long-lived effect due to integration into stem cells, and if MSCs are utilised it might offer a systemic treatment as discussed below. However, data is not yet available to support these hypotheses. It is therefore vital to perform more thorough and direct comparative studies on the different treatments options, so that patients can be better informed on the efficacy and safety of the treatments available.

8.2 SIDE EFFECTS OF GENE THERAPY

8.2.1 Insertional Mutagenesis

Gene therapy has suffered a recent setback with reports of leukaemia incidents in three of the ten children successfully treated for X-SCID (Hacein-Bey-Abina *et al.*, 2003; Check *et al.*, 2005; Kaiser *et al.*, 2005). The leukaemia, which is thought to be specific for gene therapy for X-SCID, has been found to be a direct result of the gammaretroviral integration near to and subsequent activation of the oncogene LMO2. Up till that date, this risk was recognized but was considered to be minimal as the previous 40 clinical trials involving gammaretroviral transfer to HSCs in more than 250 patients and thousands of preclinical studies in animal models had not reported the occurrence of this event (Kohn *et al.*, 2003) (although just prior to the discovery of leukaemia in the SCID patients, Li *et al.* (2002) observed one mouse with acute myeloid leukaemias from five mice that received BM transduced *ex vivo* with a gammaretrovirus).

The first case showed a monoclonal γδ T cell lymphoproliferative disorder and the gammaretrovirus was found to have integrated in reverse orientation within an intron of LMO2. The second case revealed an αβ T cell proliferation of three cell types and the gammaretrovirus had integrated 5kb upstream of LMO2. The third case involved multiple insertions affecting four different oncogenes, including LMO2, and was discovered approximately 2 years after gene therapy. LMO2 is a proto-oncogene which encodes Lim domain protein, that binds certain transcription factors required for normal haematopoiesis. LMO2 transcription is activated in childhood acute lymphoblastic leukaemia as a result of a chromosomal translocation. Transgenic mice which express Lmo2/rhombotin-2 under the transcriptional control of the CD2 enhancer/promoter have been found to develop T cell tumours as early as at 5 months of age (Larson *et al.*, 1995). In transgenic mice generated using the metallothionein promoter to direct the expression of LMO2, the number of thymocytes at the most primitive stage of differentiation increased 10-fold with thymic tumours developing in 15% of the mice suggesting that Lmo2 is specifically a T cell oncogene (Neale *et al.*, 1995). Therefore, Lmo2 is a leukaemogenic oncoprotein, however it does not cause rapid onset of leukaemia in

immunocompetent mice. LMO2 is also not one of the most commonly activated oncogenes found in spontaneous human T cell leukaemias. It is therefore possible that either LMO2 is a common retroviral integration site in $CD34^+$ cells, or random integrations activating LMO2 results in a growth advantage of those cells. The long latency (two to three years) observed in the X-SCID patients indicates that LMO2 transactivation is not sufficient to cause leukaemia and additional factors may be required. The initial mutation in progenitor cells might increase cell proliferation and therefore also the risk of accumulating second mutations.

Gamma chain cytokine signalling has been shown to enhance leukaemogenesis. IL-7 and IL-15 receptors, which contain the common gamma chain, have been implicated in T cell survival (Puel *et al.*, 1998; Schluns *et al.*, 2000; Schluns *et al.*, 2003). Transgenic mice which over express IL-7 are more susceptible to pro-B and pre-B cell tumours (Valenzona *et al.*, 1996). Those that over express IL-15 frequently develop T-NK lymphocytic leukaemias (Fehniger *et al.*, 2001). IL-7, required for thymocytes development, is of particular interest because its levels are elevated in SCID patients (Bolotin *et al.*, 1999). Therefore, increased signalling through the IL-7 receptor could have primed haematopoietic cells of SCID patients for transformation. The additional upregulation of LMO2 could multiply this effect, therefore increasing the likelihood of transforming the cells.

Hence, the activation of LMO2, which encodes a transcription factor required for haematopoiesis, in combination with the gamma chain expression, a subunit of several receptors acting as T cell growth factors, are thought to be the cause of leukaemia (Hacein-Bey-Abina *et al.*, 2003). Dave *et al.* (2004) similarly discovered murine leukaemia as a result of gammaretroviral insertions in both LMO2 and gamma c, suggesting that the two genes are cooperating to cause cancer. For this reason, the leukaemia observed should be specific to the X-SCID. Furthermore, it may be the case that a secondary event is required to activate the leukaemia, implied by the long latency of the onset of disease. In the case of the first two patients who developed leukaemia, these events could be a partial trisomy with a chromosome translocation or a varicella-zoster infection in the first patient (Hacein-Bey-Abina *et al.*, 2003; reviewed by McCormack & Rabbitts, 2004), and two chromosomal aberrations in the second patient (Hacein-Bey-Abina *et al.*, 2003). Replication

competent retrovirus was not detected in any of the three patients. The X-SCID trials which have been carried out in Great Ormond Street Hospital, London, where 8 patients have been treated, have not observed any adverse events. This could be due to a shorter follow up period than the Paris study or simply due to the fact that fewer patients have been treated. Differences in the procedure include that no FCS was used for cell culture in the London study and the gammaretrovirus was pseudotyped with the GALV envelope rather than the amphotropic envelope but it is not known whether these differences are significant. Clearly, there are other possible reasons as to why adverse events have only been observed in the Paris study and these should be further investigated to determine the risk of the gene therapy procedure.

Therefore, the leukaemia cases may be specific for X-SCID, and the risk of leukaemia as a result of ADA gene therapy is minimal. This is supported by the fact that previous gene therapy trials carried out more than a decade ago for ADA deficiency, in which patients still have significant numbers of transduced cells, and the successful ADA gene therapy trial carried out by Aiuti *et al.*, 2002a, have not observed any adverse events (5 patients have been treated in this study to date).

It is therefore important to find a balance between a high transduction efficiency, and therefore probably a high transgene expression, and achieving a copy number that reduces the risk of insertional mutagenesis. As a result of the leukaemia cases, there are extensive studies on preferential integration of gammaretroviruses and lentiviruses. Although some studies had suggested that retroviruses tend to integrate into regions of open chromatin (Vijaya *et al.*, 1986; Rohdewohld *et al.*, 1987) viral integration had previously been considered to be essentially random. It has now been demonstrated that both gammaretroviruses and lentiviruses preferentially integrate in the vicinity of transcriptionally active genes (Shroder *et al.*, 2002; Wu *et al.*, 2003). More specifically, gammaretroviral vectors were found to integrate in and around promoters of active genes, and lentiviral vectors in the transcriptional units of active genes (Bushman *et al.*, 2003; reviewed by Wu & Burgess, 2004; De Palma *et al.*, 2005).

Due to safety concerns, viral vectors are therefore constantly being developed to minimise the risk of generation of replication competent viruses, such as the

generation of split genome vectors and packaging cell lines. In any viral vector, RNA readthrough of the 3' polyadenylation signal could lead to transcription of down-stream cellular genes, including oncogenes. Hence, it will be important to develop vectors which carry an efficient polyadenylation signal to prevent these readthrough effects. Enhancers contained in viral promoters can also lead to the activation of gene transcription over large distances. The mechanism for this is not clear, but three common models have been put forward: the scanning, linking and looping models. Of these, the most accepted model is the looping model, which proposes that the contacts between enhancer bound activating proteins and promoter bound transcription factors bring together the enhancer and promoter elements, resulting in the looping out of the intervening DNA (Ptashne, 1986; Petraschek *et al.*, 2005). Insulators have therefore been used in viral vectors to prevent the unintentional activation of genes nearby to the viral integration site (Emery *et al.*, 2000; Ramezani *et al.*, 2003; Li *et al.*, 2005).

SIN-gammaretroviral vectors are currently being developed for use in gene therapy. The integrated copies of these vectors do not have functional retroviral promoters and therefore lack the enhancer activities which can lead to the activation of nearby genes. These vectors may therefore still insert in the vicinity of oncogenes, but should not lead to their activation.

More research is also currently being performed to attempt to direct the insertion of the proviral DNA into "safe" regions of the genome, including the use of the site specific integration system of bacteriophage ϕC31 (Groth *et al.*, 2000; Olivares *et al.*, 2002, Ortiz-Urda *et al.*, 2002). Urnov *et al.* (2005) recently reported the successful direct targeting and replacement of a faulty gamma chain using a different approach. The targeting was performed using zinc finger proteins to which an endonuclease was fused. The endonuclease generated a cut in the DNA, thus stimulating homologous recombination with the extrachromosomal DNA carrying the correct sequence, resulting in nearly 20% corrected cells. This technique would therefore avoid the risk of insertional mutagenesis as only the sequence of interest is targeted. Therefore, all basic research on developing vector systems is vital to avoid further adverse events such as the leukaemia cases observed in the X-SCID trial.

8.2.2 WPRE Cancer Risk

The safety of the WPRE, utilised in several vectors to enhance the transgene expression has been brought into question. A short communication regarding concerns about WPRE was recently published (Kingsman *et al.*, 2005), following reports suggesting that the WPRE may have caused liver cancer in a mouse study (Themis *et al.*, 2005a, b & c). These mice had been inoculated *in utero* using an equine infectious anaemia virus (EIAV) lentiviral vector, but the mechanism of tumour formation has still not been clarified. WPRE is considered a possible cause of the cancers because previous reports have suggested that truncated hepadna virus X proteins (the gene of which is found within the open reading frame of the WPRE) may have oncogenic properties (Sirma *et al.*, 1999; Tu *et al.*, 2001). Kingsman *et al.* (2005) reported that the EIAV vector contained an open reading frame in the WPRE which could express the truncated form of the oncogenic X protein and act as a weak oncogene. However, any link between liver cancer and the WPRE will need to be confirmed as it could in fact also have been caused by insertional mutagenesis or by other elements within the vector. In fact, of the 20 integration sites identified, half were in the vicinity of genes encoding proteins with kinase activity or nucleic acid binding functions, or proteins involved in signal transduction, transcription, cell proliferation or DNA repair, which are all important in oncogenesis. Hence, further analysis is being performed on these integration sites to elucidate the mechanism of oncogenesis.

The WPRE that may have caused liver cancer in the murine study was wild type, whereas the WPRE used in our study had been mutated in the ATG start codon of the X protein and also in the X protein promoter. These deletions are believed to minimise the risk of causing cancer, and the WPRE is therefore very unlikely to cause problems in our gene therapy trial. Importantly, lentiviral vectors containing these exact mutations were found not to result in liver cancer in this study (Themis *et al.*, 2005a, b & c). Sequencing performed on sorted T cells by Dr Zhang confirmed that the WPRE sequence in the transduced cells of patient 3 remained mutated and had not reverted back to wild type (data not shown). Therefore, the risk of liver cancer caused by the WPRE remains very unlikely.

Whilst there are possible side-effects associated with gene therapy, it is important to remember that gene therapy is still a treatment at an early stage of development, and it is crucial that clinical trials are continued so that this potentially life-saving treatment can be further developed. It is also important to realise that the patients treated with gene therapy have a life-threatening condition with limited treatment options available, which also have their own associated risks. Furthermore, at this point in time gene therapy is only a treatment choice for ADA deficiency when no bone marrow match can be found, and when PEG-ADA is not successful. Hence it offers a solution to several patients for whom other treatments are not possible or are not successful. Moreover, the overall possible risks will only become apparent after treatment of greater numbers of patients.

8.3 DEVELOPMENT OF GENE THERAPY TO ACHIEVE A SYSTEMIC TREATMENT FOR ADA DEFICIENCY

8.3.1 Lentiviral Vector as an Improved Gene Delivery System

Gene therapy currently represents a good alternative treatment for ADA deficient patients. However, improvements need to be made not only regarding the safety of the vector utilised, but protocols also need to be modified in order to achieve systemic delivery of ADA. We demonstrated that the five day activation and transduction protocol of HSCs using SFada/W gammaretrovirus decreased the expression of CD34, a marker thought to be present on most haematopoietic stem cells. Thus, if the number of stem cells were reduced during the transduction procedure, the likelihood of transducing a multipotential stem cell would clearly be reduced. It has previously been reported that the activation of $CD34^+$ cells to enter the cell cycle, necessary for the gammaretroviral vector to gain entry into the nucleus, also results in the differentiation of the $CD34^+$ cells thus reducing their multipotential nature and engraftment capacity (Bhatia *et al.*, 1997; Gothot *et al.*,1998, Rebel *et al.*, 1999). The broad differentiation potential displayed by HSCs is essential for the successful treatment of ADA deficiency, as the transduced stem cells are required to repopulate the entire haematopoietic system for effective treatment and to achieve maximal detoxification. Lentiviral vectors were therefore

considered as an alternative to the gammaretroviral vector as they are able to transduce non-dividing cells (Lewis *et al.*, 1992; Bukrinsky *et al.*, 1993; Lewis & Emerman, 1994; Naldini *et al.*, 1996a), useful for the transduction of largely quiescent cells such as HSCs and slow dividing cells such as MSCs. Lentiviral transductions of $CD34^+$ cells have been reported to require no or less activation of the cells (Uchida *et al.*, 1998; Case *et al.*, 1999; Miyoshi *et al.*, 1999; Chang *et al.*, 1999). Therefore the $CD34^+$ cells should maintain their multipotential nature and higher levels of engraftment would be expected, essential for the ADA gene therapy trial.

The ADA lentiviral vector constructed in this study was found to be more efficient than the gammaretrovirus at transducing a variety of cells, including primary skin fibroblasts, patient B-LCLs and MSCs. The lentiviral vector also efficiently transduced $CD34^+$ cells, crucially requiring less cytokines and a shorter period of activation than the gammaretrovirus. However, the multipotential nature of $CD34^+$ cells can only be demonstrated if NOD/SCID repopulating experiments are performed. The lentiviral transduced cells displayed reconstituted ADA expression and activity at levels greater than that observed in normal cells. Hence, the ADA-lentiviral vector could offer an alternative and improved vector system for use in clinical gene therapy trials.

8.3.2 Mesenchymal Stem Cells

8.3.2.1 MSC Differentiation

In the case of ADA deficiency it is likely to be important to achieve systemic delivery of ADA as it is a multi-organ disease. HSCs are one of the target cells for the improved gene therapy protocol as they are capable of differentiating into all the cells of the haematopoietic system (Hay, 1966). The additional use of mesenchymal stem cells may provide the systemic delivery method as they can differentiate into several different cell types of both mesenchymal and non-mesenchymal origin. Hence, a combination of HSCs and MSCs could be used for the gene therapy treatment of ADA deficiency to achieve haematopoietic and non-haematopoietic ADA reconstitution. MSCs have been reported to differentiate into several different

cell types, including adipocytes, osteocytes and chondrocytes (Piersma *et al.*, 1983 & 1985; Howlett *et al.*, 1986; Friedenstein *et al.*, 1987; Mardon *et al.*, 1987; Owen & Friedenstein, 1988; Keating *et al.*, 1990; Caplan, 1991; Haynesworth *et al.*, 1992; Beresford *et al.*, 1992; Cheng *et al.*, 1994.; Rickard *et al.*, 1994; reviewed by Clark & Keating, 1995; reviewed by Prockop, 1997; Pittenger *et al.*, 1999). They have furthermore been reported to differentiate into cardiomyocytes (Mangi *et al.*, 2003; Makino *et al.*, 1999; Toma *et al.*, 2002), and neuronal cells (Sanchez-Ramos *et al.*, 2000; Kohyama *et al.*, 2001; Kim *et al.*, 2002; Jiang *et al.*, 2003). The ability of MSCs to differentiate into neuronal cells was considered particularly interesting as ADA deficient children have been reported to suffer from neurological problems (Hirschhorn *et al.*, 1980; Tanaka *et al.*, 1996; Rogers *et al.*, 2001; Albuquerque *et al.*, 2004). Preliminary experiments were therefore carried out on MSCs as a potential gene delivery vehicle, as it was thought they may be able to home to areas of damage in the brain and differentiate into neuronal cells, supplying the brain with the ADA enzyme and ameliorating neurological abnormalities.

The identity of the MSCs isolated from bone marrow was verified by analysis of antigenic expression and by their *in vitro* differentiation potential. The MSCs displayed the same phenotypic characteristics as previously reported (Haynesworth *et al.*, 1992; Pittenger *et al.*, 1999; Colter *et al.*, 2000; Guo *et al.*, 2001; Suva *et al.*, 2004), including the lack of expression of the common haematopoietic markers CD34 and CD45. The ability of the MSCs to differentiate into adipocytes that accumulate lipid vacuoles, osteocytes that produce mineralized extracellular matrices, chondrocytes that produce type II collagen, and neurons that express common neuronal markers was also demonstrated under *in vitro* conditions. In fact, they retained this multipotential nature until at least passage 12. This is a significant finding as some groups have previously reported the loss of differentiation potential after extended subculture (Muraglia *et al.*, 2000; Quirici *et al.*, 2002).

The neuronal differentiation was particularly important as it demonstrated the ability of patient mesenchymal stem cells to differentiate into cells of non-mesenchymal origin, termed transdifferentiation. The expression of Tau-2, NeuN and NSE and the lack of expression of GFAP suggested that the mesenchymal stem cells had differentiated into neurons rather than astrocytes. However, to confirm the

differentiation into neuronal cells, functional and electrophysiological characteristics of the neurons would need to be demonstrated by measurement of the action potential of these cells, to ensure that the mesenchymal derived neurons have the same functional characteristics as brain derived neurons.

The multipotential MSCs were efficiently transduced by the lentiviral vector, and the ADA expression was maintained for more than four months. Transduced MSC maintained their ability to undergo adipogenic, osteogenic, and neuronal differentiation when exposed to the appropriate stimuli. Moreover, transgene expression was not affected by the differentiation into three different cell types, crucial for the application of MSC gene therapy. This has been similarly demonstrated in previous studies (Quintavalla *et al.*, 2002; Zhang, X.Y., *et al.*, 2002).

8.3.2.2 Transdifferentiation vs Fusion

Transdifferentiation, such as the differentiation of MSCs into neurons, could occur by two possible mechanisms. Transdifferentiation may be indirect, involving dedifferentiation to a more immature cell type followed by maturation down an alternative pathway, or it could be direct, in which there is a direct transition in the gene expression pattern of the cell. However, rather than differentiation, an alternative mechanism could be fusion, where the gene expression pattern of the original bone marrow stem cell is converted to that of the fusion partner. Certain scientists believe that *in vivo* experiments which have showed MSCs to be able to differentiate into neuronal cells or other cells have not observed transdifferentiation but cell fusion. Weimann *et al.* (2003a), for instance, observed that following BMT in mice, the "differentiated" cells in the brain always had 2 nuclei, one of which had originated from the bone marrow cell. They also demonstrated that the donor cells acquired the characteristics of the host cell after fusion, which would be important in the repair of neurological damage by gene or cell therapy. Previously, Prockop and colleagues (reviewed by Prockop *et al.*, 2003) also examined the possibilities of fusion and differentiation. They cultured MSCs together with heat-shocked small airway epithelial cells. The damaged epithelial cells induced the majority of the MSCs to differentiate into epithelial cells. Up to 25% of the MSCs underwent cell fusion with the epithelial cells, a few of which also underwent nuclear fusion. Hence,

as previously discussed, several groups believe to have observed MSC differentiation *in vivo* (Prockop *et al.*, 1997; Azizi *et al.*, 1998; Kopen *et al.*, 1999), however other groups report on cell fusion occurring (Wang *et al.*, 2003; Vassilopoulos *et al.*, 2003; Weimann *et al.*, 2003a and b). Therefore, there is clearly still a debate regarding the issue of differentiation versus fusion. However, it is quite likely that there is a combination of both events occurring. Ultimately, in the case of gene therapy for ADA deficiency it may not be crucial whether differentiation or fusion is occurring as the main objective is to deliver ADA expression to the brain and other organs. Hence differentiation of MSCs may not be required, and engraftment of the transduced MSCs could be sufficient. MSCs could therefore repair tissues by three different mechanisms: creating an environment that enhances regeneration of endogenous cells, transdifferentiation, or cell fusion, and either of these mechanisms would provide ADA expression to the areas of need.

8.3.2.3 Homing of MSCs

Prior to the use of MSCs in a clinical trial, the ability of MSCs to home to different tissues should be demonstrated. Therefore, the murine study in Chapter 7 was performed where MSCs were injected intravenously into sublethally irradiated NOD-SCID mice. However, no human MSCs were detected in any of the ten different organs analysed. This study would therefore need to be repeated to confirm the engraftment potential of MSCs. Reasons as to why no engraftment was observed could be due to, as previously discussed, that too few cells were injected or that the passage of the cells was too high. However, it has also been suggested that intravenous injections of MSCs may not be the ideal route of delivery as MSC do not commonly circulate in the blood stream, hence this would be an unnatural environment for the cells and they may be unable to cross the endothelial wall (Daga *et al.*, 2002). Some engraftment has been observed in a number of previous studies following intravenous injections of MSCs of conditioned mice (Dao *et al.*, 1997; Allay *et al.*, 1997; Chuah *et al.*, 2000). However this was usually detected by PCR and the percentage viability of the MSCs was not known. Thus it has been suggested that MSCs should be injected subcutaneously rather than intravenously as local delivery to the target organ may be required. Engraftment levels should therefore be assessed for the two different routes of administration in a mouse model, to

determine whether viability of the MSCs may be reduced as a result of circulation in the blood. This clearly has important implications for the use of MSCs in a human gene therapy trial.

Some observations suggest that mononuclear cells can cross the blood-brain barrier and contribute to normal turnover of microglia (Lawson *et al.*, 1992). However, bone marrow transplantations have been only partially effective in treating CNS defects (reviewed by Neufield, 1995; Peters *et al.*, 1996). Therefore, it may prove difficult to treat brain lesions effectively with either BMT or systemic administration of gene modified MSCs. This will be important to consider for future gene therapy trials using MSCs with the aim of correcting neurological abnormalities. It is vital to treat the neurological symptoms in ADA deficient children, and other symptoms such as gastrointestinal problems and immunodeficiency also need to be targeted. The route of administration is likely to be crucial to the success of systemic gene therapy, and perhaps local delivery of MSCs as well as co-injections of MSCs and HSCs intravenously may be required. However, this would be extremely difficult to perform as ADA needs to be delivered to several different organs including the brain. Intracerebral injections of MSCs have been successfully carried out in rats and mice (Schwartz *et al.*, 2001; Jin *et al.*, 2002) and MSCs injected following traumatic brain injury were found to migrate towards the site of injury (Mahmood *et al.*, 2002 & 2004). Intracerebral transplantations of foetal nerve tissue have also been carried out in human trials for Parkinson's disease although the effectiveness for this treatment has not yet been established (Widner *et al.*, 1992; Peschanski *et al.*, 1994; Levivier *et al.*, 1997; Hagell *et al.*, 1999; reviewed by Lindvall & Bjorklund, 2004). Therefore, although intracerebral injections may be difficult to perform it is clearly achievable. However, some studies (Mahmood *et al.*, 2004) suggest that MSCs injected intravenously promote cellular proliferation following traumatic brain injury in rats. Brain injury is often seen in ADA deficient patients and may therefore enhance MSC engraftment. It will therefore need to be clarified whether systemically infused MSCs can improve brain abnormalities. However, at this stage it has only been proven that ADA transduced MSCs can trans-differentiate and the next step would be to investigate models of engraftment and *in vivo* activity. Therefore, it may take some time before clinical trials using MSCs could be performed.

As well as examining engraftment levels in NOD/SCID mice, it would also be interesting to observe where the MSCs would home in an ADA deficient mouse model. It has been previously reported that MSCs preferentially home to areas of damage in animal models (Devine *et al.*, 2001; reviewed by Labat, 2001; Mahmood *et al.*, 2002 & 2004; Saito *et al.*, 2002; Shake *et al.*, 2002; Deng *et al.*, 2004). It might therefore be expected that MSCs would home to organs damaged by ADA deficiency, including the brain and the haematopoietic system which have shown abnormalities in ADA deficient children, and the GI tract which has been found to be particularly damaged in the ADA deficient mouse. This would clearly be advantageous as the MSCs would then be ideal delivery vehicles of ADA to the areas of particular need. Therefore, as well as repeating the engraftment studies in the NOD/SCID mouse model, it would be necessary to perform this study in the more relevant ADA deficient mouse model to predict where MSCs would home in an ADA deficient patient.

The study of MSC engraftment in ADA deficient mice would also need to assess whether ADA-lentiviral transduced MSCs are capable of detoxifying an entire solid organ. In current gene therapy trials for ADA deficiency, a general detoxification of the entire haematopoietic system is expected. This is because the ADA substrates are able to enter and leave the cells from the blood, hence the ADA enzyme present in the transduced cells can convert these substrates and thus reduce the levels of toxic metabolites in the entire haematopoietic system. However, in the case of a solid organ such as the liver or the brain, diffusion of toxic substrates into and out of cells may not occur. Hence, to achieve a general detoxification of the organ by a small proportion of transduced cells, metabolites would need to be carried by the organ blood vessel system between untransduced and transduced cells. However, if detoxification via the organ blood vessel system does not occur, high levels of engraftment of transduced MSCs would probably be required. The transport of metabolites in the solid organs of the ADA deficient mouse should thus be examined, and levels of metabolites should be measured in the organs following engraftment of MSCs to determine whether a general detoxification of the entire organ would be possible.

8.3.2.4 Protocol for the Use of MSCs in Gene Therapy

Engraftment studies are the starting point for important *in vivo* work that needs to be carried out prior to clinical trials using MSCs. Once the engraftment abilities of MSCs have been demonstrated, a protocol for gene therapy will need to be devised. Important points to consider are the type of conditioning required and the route of administration of the two cell types. Currently, MSCs are cultured in FCS *in vitro* as the growth of MSC has been found to be dependent on this. However, FCS should not be used in clinical trial as it carries the risks of possible infections and prions and may result in antibody formation to bovine proteins and rejection of the transplanted cells. Therefore, for clinical trials, the use of HSA or autologous serum should be further investigated as MSCs have been reported to grow well in the presence of autologous serum and retain their multipotential nature (Stute *et al.*, 2004).

The issue of PEG-ADA therapy also needs to be addressed. It is now believed that if the patient is being treated with PEG-ADA, this needs to be withdrawn prior to gene therapy in order to introduce a selective advantage to the transduced cells and maximise the chances of immune recovery. Aiuti *et al.*, (2002a) recently demonstrated that gene therapy without the use of PEG-ADA can constitute a successful treatment for ADA deficiency. In one study they successfully withdrew PEG-ADA in one patient who had previously undergone gene therapy (Aiuti *et al.*, 2002b) and in a second study PEG-ADA therapy was never initiated prior to gene therapy (Aitui *et al.*, 2002a). The successful treatment of ADA deficiency by gene therapy without the use of PEG-ADA has now been reported in five patients (Aiuti *et al.*, 2005). Therefore, our clinical gene therapy trial using gammaretroviral transduced $CD34^+$ cells included the withdrawal of PEG-ADA to maximise the selective growth advantage of transduced cells. Similarly, gene therapy using lentiviral transduced $CD34^+$ cells and MSCs would include the withdrawal of PEG-ADA. A mild conditioning was performed in the gene therapy treatment for patient 3, similar to that described in the Milan study (Aiuti *et al.*, 2002a), to create space for the transduced $CD34^+$ cells, and this would be similarly repeated for the $CD34^+$ cell/MSC gene therapy in order to encourage $CD34^+$ cell and MSC engraftment.

It has been reported that MSCs can serve as a feeder layer for HSCs *in vitro* (Dexter & Testa, 1976; Gartner & Kaplan, 1980; Quesenberry & Lowry, 1992; Sutherland *et*

al., 1993; reviewed by Deryugina & Muller-Sieburg, 1993; Majumdar *et al.*, 1998; Haynesworth *et al.*, 1996; Gordon *et al.*, 1996; Dormady *et al.*, 2001) and support the engraftment of HSCs *in vivo* in animal models (Nolta *et al.*, 1994 & 2002: Brouard *et al.*, 1998; Noort *et al.*, 2002: Bensidhoum *et al.*, 2004) and in humans (Koc *et al.*, 1999, 2000, 2001). Hence, to maximise the engraftment of HSCs and MSCs, co-injections of MSCs and HSCs would be necessary. Therefore, if clinical trials are to be carried out using MSCs and HSCs, several issues such as these will need to be examined whilst devising the gene therapy protocol.

8.3.2.5 Identification of Primitive MSCs

Further studies should be performed to identify the most primitive and multipotential MSC which could be of even more use in gene therapy for ADA deficiency than the MSCs currently being studied. It has been suggested that multipotent adult progenitor cells (MAPCs) are more immature and demonstrate greater plasticity than the MSCs (Jiang *et al.*, 2002). MAPCs do not only differentiate into MSCs but also into cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*. When transplanted, they also differentiated into HSCs as well as epithelium of the liver, lung and gut. If it is confirmed that this cell type is capable of differentiating into HSCs then MAPCs may be the only cells required for the systemic gene therapy treatment of ADA deficiency. It will therefore be important to definitively identify the most multipotential mesenchymal stem cell. The engraftment potential of these cells should also be assessed following different routes of administration, as the identification of a multipotential cell capable of engrafting into several tissues following intravenous administration is pivotal to the success of a systemic treatment for ADA deficiency.

8.3.2.6 Umbilical Cord Blood MSCs

Several studies have been carried out on MSCs isolated from umbilical cord blood (UCB). Their existence in UCB is still being debated (Wexler *et al.*, 2002), however, several groups have reported the successful isolation of multipotential MSCs from UCB (Erices *et al.*, 2000; Romanov *et al.*, 2003; Lee O.K. *et al.*, 2004; Lee, M.W. *et al.*, 2004). MSC from UCB are believed to have a wider differentiation potential as

they are more immature cells. Hence, the use of these cells could prove to be valuable if the ADA deficient child is diagnosed prenatally. The advantage of using UCB MSCs is that the child could be treated at a young age which may improve the chances of ameliorating neurological and other abnormalities before irreversible damage occurs due to continual ADA deficiency, and furthermore the risk of developing infections would be much reduced. Moreover, PEG-ADA may not need to be administered if the child is treated immediately with gene therapy, hence maximising the selective growth advantage of the transduced cells. In the case of patient 3 who was treated with gene therapy in this study, the persistent EBV infection may have been avoided had he received gene therapy earlier. Moreover, as a result of the poor response to PEG-ADA for a period of time prior to gene therapy, thymic function may have diminished and compromised immune recovery. The gene therapy treatment may therefore have been more effective if the patient was treated at a younger age, and if the disease was diagnosed prenatally UCB MSCs could have potentially been used.

8.4 *IN UTERO* CELL AND GENE THERAPY

If ADA deficiency was diagnosed pre-natally, treating ADA deficient patients prior to disease onset could be done by *in utero* cell or gene therapy. Although not proven, it is very likely that the lack of ADA during development results in considerable damage to different organs of the foetus. With conventional gene therapy, usually performed when the child is a few months old, damage to tissues might already have occurred due to the lack of ADA during gestation and the immediate post-natal period. However, with *in utero* cell or gene therapy, the foetus would develop with ADA expression is some cells during the latter part of gestation and the post-natal period. This may therefore avoid damage to the child as a result of lack of ADA during gestation and the early part of its life that might occur with conventional gene therapy. Advantages of *in utero* cell or gene therapy therefore include the possible prevention of early disease related tissue damage, the possibility of postnatal tolerance to the vector and transgene due to the underdeveloped immune system in the early stages of development (although one would have to ensure that vector tolerance does not compromise the immune defence against the wild type virus), and the possibility of targeting early stem cells believed to be highly plastic. However, with *in utero* gene therapy there is a major concern about the possibility of

inadvertent germ line modification (reviewed by Coutelle *et al.*, 2003; by Jones, 2004; and by Chauhan *et al.*, 2004). Vector DNA has been detected by PCR in the gonads of foetal sheep treated with *in utero* gene therapy, however, no germ line transmission could be found by PCR analysis of sperm derived from three rams born after breeding of *in utero* treated animals (Porada *et al.*, 2004). Due to the concerns of germ line modifications, direct injection of the transgene carrying vector is currently not permitted and is unlikely to be so for some period of time according to the Gene Therapy Advisory Committee (GTAC), although injection of non-transduced or transduced cells could be possible.

The administration of MSCs and HSCs to the foetus should be relatively straight-forward as the foetal circulation can be accessed trans-abdominally under ultrasound from 17 weeks gestation. The risk of germ-line transmission at this stage would be similar to conventional gene therapy as compartmentalisation of primordial germ cells occurs at week 7. *In utero* gene therapy has been performed in mice and sheep (Porada *et al.*, 2004 & 2005; Dejneka *et al.*, 2004; Gregory *et al.*, 2004; Shen *et al.*, 2005), and with some success in a small number of trials during human pregnancies including for X-SCID and Osteogenesis Imperfecta (Wengler *et al.*, 1996; Le Blanc *et al.*, 2005). *In utero* gene therapy using MSCs in a sheep model resulted in low level multiorgan engraftment (Schoeberlein *et al.*, 2005). The *ex vivo* gene therapy protocol currently utilised for post-natal gene therapy might be difficult to use for *in utero* gene therapy as autologous cells could be difficult to obtain. However, the reported existence of MSCs in first trimester foetal blood may make *in utero* gene therapy with MSCs possible (Campagnoli *et al.*, 2001 & 2002). If autologous cells are difficult to obtain, another option is to perform *in utero* cell therapy, which would not carry the risks of germ-line transmission (reviewed by Flake, 2004). This would involve the administration of MSCs from a normal donor which would provide the foetus with ADA positive cells thus avoiding the need for transducing the cells with an ADA vector. However, this would clearly then rely on finding a matched donor, which is usually only available for one third of the patients.

In utero cell or gene therapy for ADA deficiency should be tested extensively in animal models prior to the initiation of clinical trials. It would be interesting to perform studies in ADA deficient mice, comparing immunological and non-

immunological symptoms in groups of mice that received either *in utero* cell or gene therapy or post-natal cell or gene therapy. It would be expected that the mice receiving *in utero* therapy would sustain less damage to different organs and show a better immune recovery. However, the significance of the results of these studies would be limited by the fact that mice display an even stronger requirement for ADA during gestation than humans, as ADA deficient mice have been found to die peri-natally. Differences in response might therefore be observed in ADA deficient mice and humans treated with *in utero* therapy. Nevertheless, it would still be necessary to perform these murine studies and more pre-clinical experiments to determine whether there is a risk of germ line transmission prior to performing *in utero* gene therapy in humans. Therefore, this could be another step forward in the treatment of ADA deficiency as it may be crucial to deliver ADA at the earliest stage possible to minimise the damage to different organs as a result of an absence of ADA.

8.5 CONCLUDING REMARKS

This study has highlighted that PEG-ADA therapy can be a successful treatment for some ADA deficient patients. For others, however, gene therapy is a good alternative treatment when a fully matched bone marrow donor is not available and when PEG-ADA therapy is ineffective. Current gene therapy trials involve the use of transduced $CD34^+$ stem cells, which have proven to successfully treat the immunological symptoms of ADA deficiency. However, lentiviral transduced MSCs together with $CD34^+$ cells could constitute a more systemic treatment for ADA deficiency than gene therapy using $CD34^+$ cells alone. The main findings of this study were:

- PEG-ADA therapy is a successful treatment for some ADA deficient patients.
- The SFada/W gammaretroviral vector efficiently transduced a number of cell types, including patient primary skin fibroblasts and haematopoietic stem cells, resulting in enhanced ADA expression and activity.
- Gene therapy using $CD34^+$ cells improved the immunological function of one ADA deficient patient for whom PEG-ADA therapy was not successful.
- The ADA lentiviral vector was more efficient than the gammaretroviral vector at transducing several cell types, including patient primary skin

fibroblasts and in particular B-lymphoblastoid cells and mesenchymal stem cells.

- Lentiviral transductions resulted in enhanced ADA expression in haematopoietic stem cells, and enhanced ADA expression and activity in fibroblasts, B-LCLs and MSCs.
- Mesenchymal stem cells were readily isolated from bone marrow and their multipotential nature was demonstrated *in vitro* by differentiation into four different cell lineages.
- The multilineage potential of MSCs was maintained following lentiviral transductions and transgene expression was retained following differentiation.

8.6 FUTURE WORK RELATED TO THIS STUDY

Further work in relation to this study is listed below:

- Evaluate the WPRE to confirm that the WPRE does indeed increase transgene expression and does not constitute a cancer risk.
- Identify the most multipotential stem cell, possibly MAPCs or UCB MSCs.
- Perform functional studies on differentiated MSCs including measurement of action potential of MSC derived neurons to confirm that MSC derived neurons are functional.
- Confirm the homing abilities of MSCs in the NOD/SCID mouse model.
- Perform engraftments of transduced MSCs and $CD34^+$ cells to study MSC support of HSC engraftment and to ultimately rescue the ADA deficient mouse.
- Perform further assessments of ADA deficient patients treated with PEG-ADA, BMT and gene therapy to identify which treatment results in better immune reconstitution, improvement in non-immunological abnormalities and overall systemic detoxification.

8.7 PUBLICATIONS RELATED TO THIS STUDY

Bjorkegren, E.K.M., Parsley, K.L., Fairbanks, L.D., Gilmour, K.C., Kuehlcke, K., Kinnon, C., Thrasher, A.J., Gaspar, H.B (2003). Development of Gene Therapy Vectors for the Treatment of Adenosine Deaminase (ADA)-Deficient Severe Combined Immunodeficiency (SCID). Poster presentated at the *European Society of Gene Therapy Annual Meeting, Edinburgh.*

Gaspar H.B, Bjorkegren E, Parsley K, Gilmour K.G, Fairbanks L, King D *et al.,* (2004). Somatic gene therapy for ADA SCID following cessation of PEG-ADA and use of a mild conditioning regime. Presented at *11th meeting of the European Society of Immunodeficiencies, Versailles, France.*

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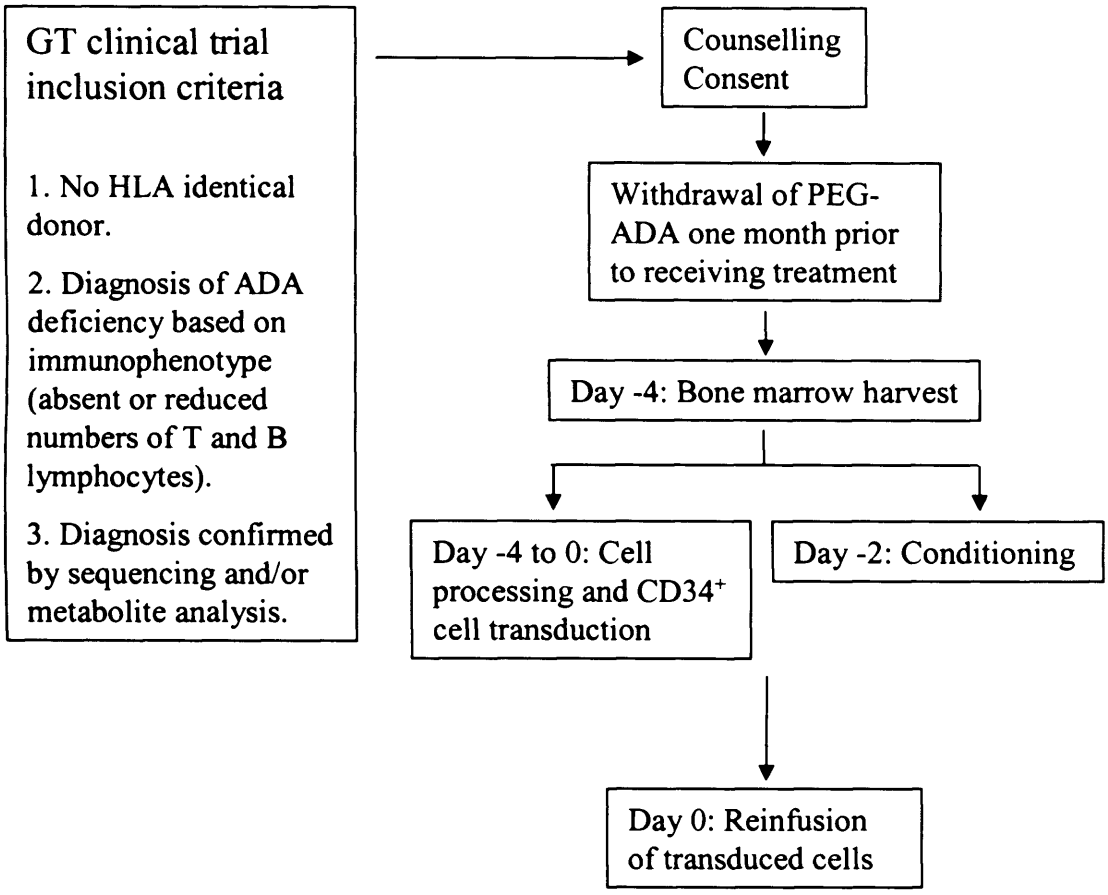
Lymphocyte subpopulations	Neonatal	1wk-2mo	2-5mo	5-9mo	9-15mo	15-24mo	2-5yr	5-10yr	10-16yr	Adults
Lymphocytes	4,800	6,700	5,900	6,000	5,500	5,600	3,300	2,800	2,200	1,800
$CD19^+$ B lymphocytes	600	1,000	1,300	1,300	1,400	1,300	800	500	300	200
$CD3^+$ T lymphocytes	2,800	4,600	3,600	3,800	3,400	3,500	2,300	1,900	1,500	1,200
$CD3^+/CD4^+$ T lymphocytes	1,900	3,500	2,500	2,800	2,300	2,200	1,300	1,000	800	700
$CD3^+/CD8^+$ T lymphocytes	1,100	1,000	1,000	1,100	1,100	1,200	800	800	400	400
$CD3^+/CD16\text{-}56^+$ NK cells	1,000	500	300	300	400	400	400	300	300	300

Appendix 1. Absolute size of lymphocyte subpopulations[a] at different ages. Adapted from Comans-Bitter *et al.*, 1997.
[a] Absolute counts (/μL).



Appendix 2. Gene therapy protocol.